UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA VETERINÁRIA

CLINICAL AND MOLECULAR CHARACTERIZATION OF FELINE MAMMARY CARCINOMAS OVEREXPRESSING HER2 PROTO-ONCOGENE (FMC-HER2+) – NEW STRATEGIES FOR EFFECTIVE DIAGNOSTIC AND CANCER THERAPY

Maria João da Costa Soares da Silva

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 de Ciências Biológicas e Biomédicas

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Para os meus pais, Maria Custódia e César Soares
por serem os meus maiores mentores e o meu maior e mais bonito exemplo de Vida

Para o Tiago Silva,
porque és o meu anjo aqui na Terra

Para o meu irmão, Pedro Soares,
porque te preocupas

“Cancer, unlike politics and religion, is not a topic of controversy. No one is for it. Cancer is not another word for death. Neither is it a single disease for which there is one cure. Instead, it takes many forms, and each form responds differently to treatment.”

Samantha Mooney
In “A snowflake in my hand”

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ABSTRACT

Considering the scarce data available in feline mammary carcinoma (FMC) and despite its importance in veterinary clinical practice, this thesis emerges in order to increase the knowledge of this tumor type, especially the FMC-HER2 positive.

In the first two studies, the protocols for detection and quantification of the *f*HER2 and Ki-67 biomarkers were optimized and validated. These studies demonstrated that, in cats, the incidence of *f*HER2 overexpression were similar to women (about 30%), although no gene amplification was detected. It was also demonstrated that high levels of Ki-67 index were associated with a worse prognosis.

Using a panel of protein biomarkers, the FMC were divided into six different groups that demonstrated prognostic value, similarly to what is described in women. In fact, cats with triple negative basal-like or HER2-positive subtypes were associated with shorter overall survival, contrasting with cats presenting luminal A tumors. Moreover, these studies also indicated that luminal B and triple negative basal-like subtypes are the most common in cats.

When the metastatic lesions were evaluated, a marked loss of receptor expression was found, which was associated with an increase of the triple negative basal-like subtype, highlighting the importance of immunophenotyping all lesions (primary and metastatic) in cats. Considering these results, the development of diagnostic methodologies that allows the continuous follow-up of the patients would be very useful. Therefore, the last study presented in this thesis evaluates the *f*HER2 serum levels in cats with FMC using two different immunoassays (ELISA and dot blot). The serum levels of *f*HER2 were significantly associated with the *f*HER2 in tissue samples of FMC (assessed by IHC). This is consistent to what is described for humans and suggests that serum quantification could be an important tool for monitoring cats after the surgery.

In sum, the results presented herein provide new diagnostic and prognostic tools for veterinary oncology. Considering the high prevalence and similarities with the human counterpart, cat can also represent a potential animal model for the study of luminal B and triple negative subtypes. Considering *f*HER2-positive FMC more studies are required in order to determine the aetiology of the protein overexpression.

Keywords: feline mammary carcinoma; molecular classification; HER2; metastatic disease; prognostic value.

RESUMO

Os tumores mamários felinos (TMF) são umas das neoplasias mais comuns em Oncologia Felina, com uma incidência que pode atingir os 40%, pelo que assumem um papel relevante na prática clínica veterinária. Estes tumores apresentam habitualmente uma etiologia maligna (carcinomas) e um comportamento agressivo, estando associados a um prognóstico reservado. Atualmente, existem poucas opções terapêuticas que permitam aumentar a qualidade e a esperança média de vida dos animais afectados por esta neoplasia. Assim, estudos que permitam uma melhor caracterização dos tumores, identificando potenciais biomarcadores que possam ser utilizados como factores de prognóstico ou preditivos, são fundamentais para o desenvolvimento da Medicina Felina. Por outro lado, os carcinomas mamários que ocorrem espontaneamente nos animais de companhia têm sido sugeridos como potenciais modelos biológicos para o estudo do Cancro da Mama, com vantagens, comparativamente aos animais de laboratório que são atualmente utilizados. De facto, nesses animais, os tumores são quimicamente induzidos ou xenotransplantados, pelo que são considerados modelos mais artificiais. Deste modo, esta tese de doutoramento surge com o objetivo de aumentar o conhecimento sobre os tumores mamários felinos, com especial interesse no recetor transmembranar para o fator de crescimento epidérmico de tipo 2 (HER2), quer numa perspetiva clínica, de forma a melhorar a qualidade de vida destes animais de companhia, abrindo portas a novos meios de diagnóstico e potenciais novos alvos terapêuticos, quer no sentido de investigar a viabilidade de a Gata ser um bom modelo animal para o estudo do Cancro da Mama na Mulher.

Relativamente à Mulher, a investigação oncológica desenvolveu grandes esforços para encontrar biomarcadores que permitam otimizar o diagnóstico e o tratamento do cancro da mama. Entre estes, encontra-se a proteína HER2, uma oncoproteína que pode estar sobreexpressa em vários tumores da espécie humana (mama, pâncreas, cólon, próstata, bexiga, entre outros) e que lhes confere elevada agressividade e prognóstico reservado.

No Cancro da Mama, estima-se que entre 10 a 40% dos tumores apresentem amplificação do gene HER2, o que se traduz na sobreexpressão da proteína. Estas alterações são rotineiramente detetadas através de duas técnicas moleculares, a hibridação *in situ* (ISH) e a imunohistoquímica (IHC), respetivamente. A avaliação do *status* da proteína HER2 é importante, não só pelo seu valor de prognóstico, mas também como fator preditivo, já que pacientes com sobreexpressão desta proteína são elegíveis para tratamento com terapêuticas específicas dirigidas contra o HER2, tais como os anticorpos anti-HER2 (sendo o trastuzumab o mais conhecido), o que veio aumentar consideravelmente a sobrevida destas doentes.

Contrastando com a medicina humana, a literatura disponível em medicina veterinária apresenta ainda escassos estudos sobre a importância da proteína HER2 nos TMF, ou

sobre os novos sistemas de classificação que subdividem os tumores mamários conforme o seu perfil imunofenotípico.

Assim, os primeiros estudos apresentados nesta tese permitiram a otimização do protocolo de deteção da proteína *f*HER2 através de IHC, de onde se concluiu que existe uma percentagem relevante de TMF que sobreexpressam *f*HER2 (cerca de 30%), semelhante à incidência descrita na Mulher. Para além deste dado, estes estudos indicaram que a sobreexpressão do *f*HER2 não tem origem na amplificação do gene felino, demonstrando que a sobreexpressão proteíca terá uma etiologia diferente da que está descrita para a Mulher.

Durante o segundo estudo, em que foi identificado o melhor valor de referência (cut-off) para a avaliação do índice de Ki-67 nos TMF, foram estabelecidas associações com a proteína *f*HER2. O índice de Ki-67 é um importante biomarcador de proliferação celular em medicina humana, com valor de prognóstico. Adicionalmente, este índice também é importante nos sistemas de classificação molecular dos tumores mamários, uma vez que permite diferenciar os tumores de subtipo luminal A e luminal B. Foi ainda estabelecido como cut-off para o índice de Ki-67 o valor de 14%, que é o cut-off mais utilizado em medicina humana. Foi ainda observado que o índice de Ki-67 apresenta valor de prognóstico nos TMF, uma vez que gatas com tumores mamários com elevados índices de Ki-67 (≥14%) foram associadas a uma diminuição do tempo de sobrevida e do tempo livre de doença. Em relação ao *f*HER2, foi demonstrada uma associação com índices mais baixos de Ki-67 (<14%) e, portanto, com apresentações clínicas menos agressivas o que, aparentemente, contradiz o descrito em medicina humana.

O terceiro estudo caracteriza uma população de TMF (n=229) diagnosticados num grupo com cerca de 100 gatas, de acordo com a classificação molecular recomendada por um painel internacional de especialistas (St Gallen International Expert Consensus Panel), dividindo-a em 6 subtipos moleculares: luminal A, luminal B/HER2-negativo, luminal B/HER2-positivo, HER2-positivo, triplo negativo do tipo basal e triplo negativo do tipo normal. Neste estudo, o subtipo mais comum foi o luminal B (em primeiro o luminal B/HER2-negativo e em segundo o luminal B/HER2-positivo), sendo seguido pelo triplo negativo do tipo basal, o que difere com o descrito em medicina humana, onde o subtipo luminal A é o mais comum. O estudo demonstrou ainda que, à semelhança da Mulher, o subtipo luminal A encontrou-se associado a características mais benignas, em oposição ao subtipo triplo negativo do tipo basal, que foi associado às características tumorais mais agressivas, tais como tumores de maiores dimensões, com maior grau de malignidade, presença de necrose tumoral e maior índice de Ki-67. Relativamente ao *f*HER2, este estudo demonstrou que a grande maioria dos tumores que sobreexpressam o *f*HER2 pertencem ao subtipo luminal B/HER2-positivo (cerca de 84% dos tumores *f*HER2 positivos) e não ao subtipo HER2-positivo, o que poderá justificar as associações encontradas no estudo anterior (capítulo II) e

no último estudo (capítulo V), já que o subtipo luminal B/HER2-positivo não apresenta características tão agressivas como o subtipo HER2-positivo. De facto, a análise de sobrevivência univariada e multivariada demonstrou que, à semelhança do que está descrito na Mulher, os subtipos triplo negativo do tipo basal e HER2-positivo apresentam menores tempos de sobrevida, em comparação com os outros subtipos moleculares, de onde o luminal A é o que apresenta melhor prognóstico.

Neste trabalho de doutoramento, foram também avaliadas e classificadas as lesões primárias e metastáticas de 23 animais que morreram no decurso do estudo, em consequência da progressão da doença oncológica. Em relação à distribuição das metástases distantes, o pulmão foi o orgão mais afetado, sendo seguido pelos linfonodos não regionais e pelo fígado. Foi ainda observada uma elevada heterogeneidade entre o perfil molecular dos tumores primários e das lesões metastáticas, com uma clara tendência para a perda da expressão dos biomarcadores estudados (*f*HER2, recetor do estrogénio e recetor da progesterona) e um consequente aumento do subtipo triplo negativo do tipo basal nas metástases dos animais avaliados. Tal como havia sido observado no segundo estudo, o índice de Ki-67 demonstrou uma tendência para aumentar os seus níveis de expressão nas lesões metastáticas comparativamente aos tumores primários. Desta forma, a alteração do perfil molecular no decurso da progressão da doença metastática exige que todas as lesões (primárias e secundárias) sejam avaliadas, de forma a delinear o melhor protocolo terapêutico, sobretudo no caso do desenvolvimento de terapêuticas dirigidas para estes animais. Isto constitui uma importante limitação, uma vez que a caracterização molecular das lesões secundárias pode ser inviável (dependendo da localização da metástase e do estado clínico do animal) e corresponde a um acréscimo nos custos do maneio da doença. Assim, a utilização de outros métodos de diagnóstico que permitam o controlo da doença de forma menos invasiva, mais acessível e mais rápida, tais como a determinação dos níveis séricos de biomarcadores, poderá ser uma alternativa interessante para o seguimento de gatas com tumores mamários.

No último estudo, os níveis de *f*HER2 sérico foram determinados, utilizando duas técnicas, o ELISA e o dot blot. Foi estabelecido como valor de referência a concentração de 10 ng/ml e os níveis séricos de *f*HER2 foram positivamente associados a animais com TMF *f*HER2-positivos, o que é idêntico ao descrito para a mulher. Tendo em conta a elevada discordância entre a expressão de *f*HER2 em tumores primários e metástases, o desenvolvimento destas técnicas poderá ser importante no maneio do paciente felino após a cirurgia, sendo necessários mais estudos, de forma a perceber se os níveis séricos de *f*HER2 podem ser correlacionados com a progressão da doença metastática ou com a resposta ao tratamento, sobretudo se forem utilizadas terapêuticas dirigidas contra o *f*HER2.

Em conclusão, os estudos aqui apresentados fornecem metodologias otimizadas para avaliação de biomarcadores em Oncologia Felina e o estudo prospetivo permitiu a

determinação de novos fatores de prognóstico em carcinomas mamários felinos, o que abre portas a novos estudos, quer no sentido de melhorar e implementar novas metodologias de diagnóstico, quer no sentido do desenvolvimento de novas abordagens terapêuticas. Paralelamente, estes resultados podem ainda ser úteis em oncologia comparada, uma vez que os TMF apresentam várias semelhanças com os tumores de mama na mulher. Desta forma, as gatas com TMF poderão constituir um bom modelo animal para o estudo de tumores de mama, sobretudo do subtipo luminal B e triplo negativo, que são os subtipos mais resistentes e com menos terapêuticas efetivas em medicina humana.

Palavras chave: tumores mamários felinos; classificação molecular; recetor transmembranar para o fator de crescimento epidérmico de tipo 2 (HER2); doença metastática; valor de prognóstico.

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LIST OF ABREVIATIONS

aa – amino acids
ADAM – A Disintegrin and Metalloproteases
ADCC – Antibody-dependent cellular cytotoxicity
ADCs - Antibody-drug conjugates
AgNOR – Argyrophilic nucleolar organiser regions
AI – Aromatase inhibitors
ADR – Androgen receptor
AgR – Antigen retrieval
Akt – see PKB
ANOVA – Analysis of variance
AR – Amphiregulin
ASCO – American Society of Clinical Oncology
ATP – Adenosine triphosphate
AUC – Area under the curve
AVMA – American Veterinary Medicine Association
bp – base pair
Bcl-2 – B cell lymphoma 2
BIRC5 – Survivin or Baculoviral inhibitor of apoptosis repeat-containing 5
BRCA1 – Breast cancer susceptibility 1
BRCA2 – Breast cancer susceptibility 2
BSA – Bovine serum albumin
BTC – Betacellulin
cDNA – complementary Deoxyribonucleic acid
CEP – centromere evaluation probe
CI – Confidence interval
CISH – Chromogenic *in situ* hybridization
CK – Cytokeratin
CNS - Central Nervous System
COX-2 – Cyclooxygenase-2
CT – Computed tomography
CTCs – Circulating tumor cells
CTF – Carboxyl-terminal fragment
DAB – diaminobenzidin-tetrahydrochlorid
DAPI – 4’, 6-Diamidino-2-phenylindole, dihydrochloride
DEP-1 – Density-enhanced phosphatase-1
DFS – Disease free survival
DM – Distant metastases
DM1 – Maytansine
DNA – Deoxyribonucleic acid
DSH – domestic shorthaired
e.g. – *exempli gratia*
ECD – Extracellular domain
EDTA – Ethylenediamine tetraacetic acid
EE – Elston & Ellis
EGF – Epidermal growth factor
EGFR – Epidermal growth factor receptor
EGR – Epidermal growth receptor
ELISA – Enzyme-Linked Immunosorbent Assay
EMT – Epithelial to mesenchymal transition
EPG – Epigen
EPR – Epiregulin
ER – Estrogen receptor
Erk – Extracellular signal-regulated kinases

FDA – Food and Drug Administration
FFPE – formalin-fixed paraffin-embedded
*f*HER2 – feline epidermal growth factor receptor type 2
FISH – Fluorescence *in situ* hybridization
FITC – fluorescein isothiocyanate
FMC – Feline mammary carcinomas
FMT – Feline mammary tumors
FMV – Faculty of Veterinary Medicine
FNA – Fine-needle aspiration
FOXA1 – Forkhead box protein A1
FOXM1 – Forkhead box protein M1
*f*TOP2A – feline topoisomerase II alpha gene
GATA3 – GATA-binding protein 3 or Trans acting T-cell specific transcription factor
GGI – Genomic Grade Index
GRB7 – Growth factor receptor-bound protein 7
H&E – Hematoxylin & eosin
HB-EGF – Heparin-binding EGF-like growth factor
HDACi – Histone deacetylase inhibitor
HE – Hematoxylin & Eosin
HER - Epidermal growth receptor
HER1 – Epidermal growth factor receptor
HER2 – Human epidermal growth factor receptor-2
HER2+ - HER2-positives
HER2- - HER2 negative
HER2-ECD – extracellular domain fragment of HER2
HER3 – Human epidermal growth factor receptor-3
HER4 – Human epidermal growth factor receptor-4
HP – Histopathological
HPF – High power fields
HSP90 – Heatshock protein-90
HR – Hazard ratio
HRP – Horseradish peroxidase
HRs – Hormone receptors
I.C.V.G.A.N. – International Committee on Veterinary Gross Anatomical Nomenclature
ICD – Intracellular domain
i.e. – *id est*
IGF – Insulin-like growth factor
IgG – Immunoglobulin G
IHC – Immunohistochemistry
IMM – Instituto de Medicina Molecular
ISH – *in situ* hybridization
JAK – Janus kinase
kDa – kilo Dalton
L – Ligand
Lc. a – Axillary lymph center
Lc. I – Inguinalfemoral lymph center
LI – Lymphocitic infiltration
LNS – Lymph node status
LOH – Loss of heterozygosity
LR – Local relapse
M – Metastasis
mAbs – monoclonal antibodies
MAPK – Mitogen-activated protein kinase
MBC – Metastatic breast cancer
MC – Mammary carcinoma
MCh – Mastectomy plus chemotherapy
MES – 2-[*N*-morpholino]ethanesulphonic acid buffer

MG – malignant grade
MLPA – Multiplex ligation-depent probe amplification
MMP – Matrix Metallo Proteases
mRis – microRNAs
mRNA – messenger ribonucleic acid
MT – mammary tumors
mTOR – mammalian Target of rapamycin
MW – Microwave
N – Lymph node
NFC – Norwegian Forest Cat
NPV – Negative predictive value
NRG – Neuregulins
O/N – overnight
OR – Odds ratio
OS – Overall survival
p-AKT – Phosphorylated protein kinase B
PARP-1 – poly-ADP ribose-polymerase-1
PBS – Phosphate buffered saline
PC – pressure chamber
PCNA – Proliferating cell nuclear antigen
PCR – Polymerase chain reaction
PI3K – Phosphatidylinositol 3 kinase
PI3K-PKB/Akt – Phosphatidylinositol 3 kinase-protein kinase B
PIK3CA – Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha isoform
PLC-PKC – Phospholipase C-protein kinase C
PKB – Protein kinase B
PPV – Positive predictive value
PR – Progesterone receptor
PT – Primary tumors
PTEN – Phosphatase and tensin homolog
PTP1B – Protein tyrosine phosphatase-1B
qPCR – Quantitative polymerase chain reaction
qRT-PCR – Quantitative real time reverse transcript polymerase chain reaction
Ras-MAPK – Ras-mitogen-activated protein kinase
RE – Endoplasmatic resticulum
rHER2-ECD – recombinant human HER2-ECD
RIPA – Radioimmunoprecipitation assay
RM – Regional metastases
RNA – Ribonucleic acid
ROC – Receiver-operating characteristics
RR – Relative risk
RT – room temperature
RT-PCR – Reverse transcription polymerase chain reaction
RTU – ready-to-use
SC – Squamous cell
SD – Standard deviation
SDS – Sodium deoxycolate
SDS-PAGE – Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SERMs – Selective estrogen receptor modulators
sHER – serum HER2
SISH – Silver-enhance *in situ* hybridization
SPSS – Social Package for Social Sciences
SSC – Saline sodium citrate
STAT – Signal transducer and activator of transcription
T - Tumor
T4 – Thyroxine
T-DM1 – Ado-trastuzumab emtasine

TBS – Tris-buffered saline
TBST - Tris-buffered saline plus 0.05% Tween 20
TGF-α – Transforming growth factor-α
TK – Tyrosine kinases
TKIs – Tyrosine kinase inhibitors
TM – Transmembrane domain
TMB – 3,3′,5,5′-tetramethyl-benzidine
TMF – Tumores mamários felinos
TN – Triple Negative
TOP2A – topoisomerase II alpha gene
TP – Tubulopapillary
UK – United Kingdom
ULisboa – University of Lisbon
uPA – Urokinase-type plasminogen activator
USA – United States of America
UTAD – Universidade de Trás dos Montes e Alto Douro
v-erbB – Avian erythroblastosis virus
VEGF – Vascular endothelial growth factor
WB – water bath
WHO – World Health Organization
v. – vessels
XBP1 – X-box binding protein 1

INTRODUCTION

INTRODUCTION

THESIS OUTLINE AND OVERVIEW

"In 2010, about six hundred thousand Americans, and more than 7 million humans around the world, will die from cancer. In the United States, one in three women and one in two men will develop cancer during their lifetime. A quarter of all American deaths, and about 15 per cent of all deaths worldwide, will be attributed to cancer. In some nations, cancer will surpass heart disease to become the most common cause of death."

Siddhartha Mukherjee
In "The Emperor of all Maladies: A Biography of Cancer"

Cancer is one of the most common causes of death worldwide, with a high incidence in developed countries, despite the substantial progress on prevention, treatment and basic knowledge for cancer (Jemal *et al.*, 2011; Global Burden of Disease Cancer Collaboration, 2015; World Health Organization [WHO], 2015). This alarming advance results from the aging and growth of the world population as well as the adoption of cancer-associated risk factors, as smoking, physical inactivity/obesity and dietary patterns (Jemal *et al.*, 2011; Global Burden of Disease Cancer Collaboration, 2015). The global burden of cancer has extensive economic implications for society, added to the obvious social and individual effects (American Cancer Society, 2015). In 2012, there were estimated 14.1 million cancer cases around the world (7.4 million cases in men and 6.7 million in women) and this number is expected to increase to 24 million by 2035 (WHO, 2015).

Additionally, breast cancer represents the highest incidence in women, is the second leading cause of cancer death and foremost, this type of cancer has been continuously increasing (Global Burden of Disease Cancer Collaboration, 2015), leading to efforts for a better understanding of breast cancer biology and the development of more effective cancer treatments and prevention.

Therefore, breast cancer has been the target of several studies by scientific community. Research allowed the passage from classification systems exclusively based on morphological features to systems that include molecular markers, the development of target therapies and the improvement of the predictive and prognostic factors of the disease (Vuong *et al.*, 2014).

Human epidermal growth factor receptor-2 (HER2) is one molecular marker that has demonstrated particularly importance in breast cancer, due to its prognostic and therapeutic

implications (Benusiglio, 2007). Once breast cancer presents a well known heterogeneity, readily molecular classification systems were proposed, taking several molecular markers into account while different prognostic and predictive subgroups were determined (Gama *et al.*, 2008; Voung *et al.*, 2014).

The research work presented in this thesis aimed to evaluate the role of HER2 protein in feline mammary carcinomas (FMC), assuming this protein as a single molecular marker and also using a molecular classification system with several molecular markers usually used in breast cancer studies.

In this study, the cat was selected mainly for two main reasons. First the possibility to contribute for the advance in the understanding of breast cancer biology and to improve treatment options, prevention and quality of life of our companion animals. Secondly, to assess whether FMC could be used as animal model in breast cancer research.

The research outcome of this work resulted in five manuscripts that were published in international peer-reviewed journals and represent five chapters of the experimental work include in this thesis, as follows:

Chapter I: Feline HER2 Protein Expression Levels and Gene Status in Feline Mammary Carcinoma: Optimization of Immunohistochemistry (IHC) and *In Situ* Hybridization (ISH) Techniques

Soares, M., Correia, J., Rodrigues, P., Simões, M., de Matos, A., Ferreira, F. (2013). *Microscopy and Microanalysis,* 19(4): 876-82.

Chapter II: Ki-67 as prognostic factor in feline mammary carcinoma – what is the optimal cut-off value?

Soares, M., Ribeiro, R., Carvalho, S., Peleteiro, M., Correia, J., Ferreira, F. (2016). *Veterinary Pathology*, 53(1): 37-43.

Chapter III: Molecular based subtyping of feline mammary carcinomas and clinicopathological characterization

Soares, M., Madeira, S., Peleteiro, M., Correia, J., Cardoso, F., Ferreira, F. (2016). *The Breast Journal*, 27: 44-51.

Chapter IV: St Gallen molecular subtypes in feline mammary carcinoma and paired metastases-disease progression and clinical imlications from a 3-year follow-up study

Soares, M., Correia, J., Peleteiro, M.C., Ferreira, F. (2015). *Tumor Biology* [Epub ahead of print]

Chapter V: Serum HER2 levels are increased in cats with mammary carcinomas and predict tissue HER2 status

Soares, M., Ribeiro, R., Najmudin, S., Gameiro, A., Rodrigues, R., Cardoso, F., Ferreira, F. (2016). *Oncotarget* [Epub ahead of print]

LITERATURE REVIEW

1. Feline mammary tumors

1.1. Anatomy, histology and physiology of the normal mammary gland

As mammals, cat species (*Felis catus*, Linnaeus, 1758) have mammary glands to produce and secrete milk for the nourishment of their new born offspring. Moreover, they are also important to provide passive immunity through the colostrum (Cunningham, 2002).

Histologically, the mammary gland is classified as a modified sudoriparous apocrine gland, which is formed by several compound tubuloalveolar glands (Burkitt *et al.*, 1994). For the micro and macro development of this gland, hormones like estrogen, progesterone and prolactin are essential (Cunningham, 2002).

Typically in cats, we observe eight mammary glands arranged in two mammary chains (Figure 1), one on each side, extending from the pectoral region to the inguinal region (Raharison & Sautet, 2006). Usually they are identified as two thoracic pairs (T1, T2) and two abdominal pairs (A1, A2) of mammary glands (Raharison & Sautet, 2006) or as thoracic (one pair), abdominal (two pairs, cranial and caudal) and iguinal (one pair) mammary glands (Dyce *et al.*, 2010). Occasionally, accessory mammary glands can be present (Raharison & Sautet, 2006).

According to the vascularization, the mammary glands are supply through the follow arteries (Figure 1A): T1 and T2 mammary glands are irrigated by lateral thoracic and intercostal vessels (laterally), and internal thoracic vessels (medially), A1 mammary gland is irrigated by epigastric cranialis superficialis vessels, and A2 mammary glands receive blood from epigastric caudalis superficialis arteries (König & Liebich, 2004; Giménez *et al.*, 2010). The veins of cat mammary glands closely follow the arteries, except some small veins that cross the mideline and are thought to be responsible for the spread of malignant cell tumors between the contralateral mammary glands (Giménez *et al.*, 2010). Similarly, the venous vascularization of the thoracic glands through the chest wall (via the internal thoracic or the intercostal veins), could allow the tumor spreading to thoracic cavity (Silver, 1966).

Finally, the lymphatic drenage of mammary glands are the major responsible of the spread of neoplastic cells with origin in this organ. The two thoracic mammary glands lymphatic drainage comprises the axillary lymph center (composed by the axillary of first rib lymph node, proper axillary lymph node and accessory axillary lymph node) and the sternal cranial

lymph node. The caudal epigastric lymph nodes and the superficial inguinal lymph node, that constitute the inguinalfemoral lymph center are responsible for the lymphatic drainage of the two abdominal mammary glands (Raharison & Sautet, 2006; Raharison & Sautet, 2007; Dyce & Wensing, 2010; International Committee on Veterinary Gross Anatomical Nomenclature [I.C.V.G.A.N.], 2012). Conversely to the venous circulation, the lymphatic vessels do not cross the midline (Raharison & Sautet, 2007) but the T1 and A2 mammary glands (Figure 1) drain for the axillary and inguinalfemoral lymph centers, which is important for surgery management (Raharison & Sautet, 2006).

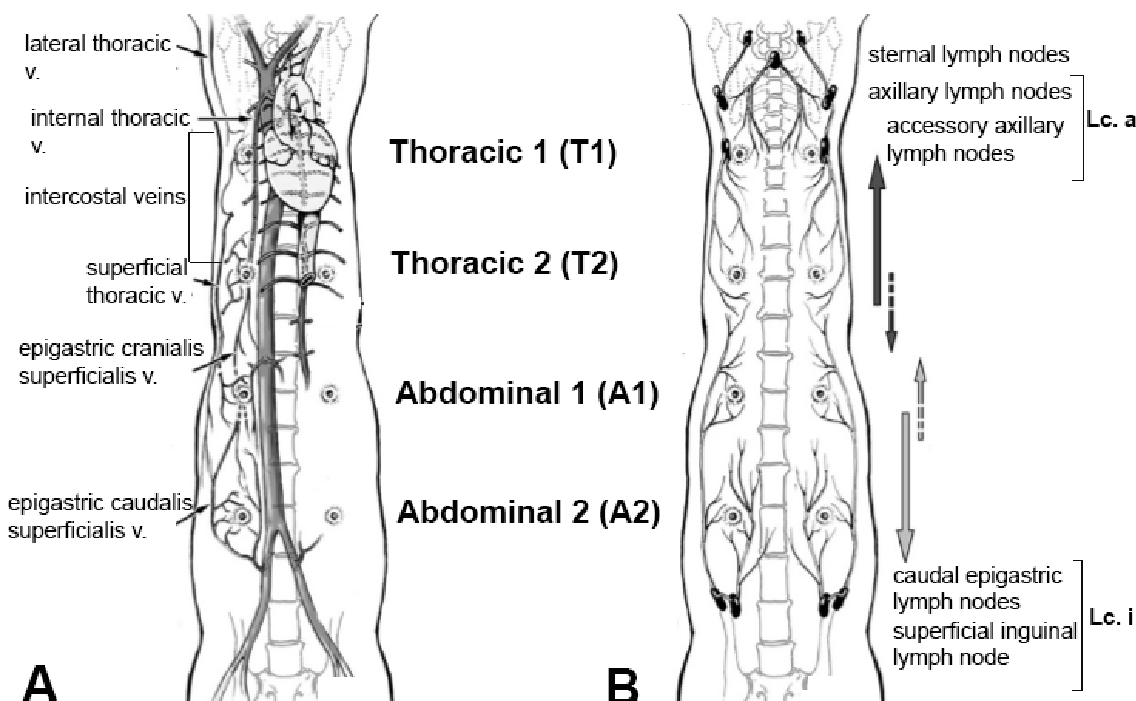

Figure 1. Schematic representation of mammary glands in cat

A, The four pairs of mammary glands in the cat and the corresponding irrigation. **B,** General schematic drawing of the lymphatic drainage of the glands and their respective lymph nodes. The large black arrow represents the drainage of T1 and T2 for the axillary lymph center and the large grey arrow represents the drainage of A1 and A2 for the inguinalfemoral lymph center, whereas the small black arrow represents the drainage of T2 to the inguinalfemoral lymph center and the small grey arrow represents the drainage of A1 for the axillary lymph center. v., vessels; Lc. a, axillary lymph center; Lc. I, inguinalfemoral lymph center. *Adapted from* Raharison & Sautet (2006); Raharison & Sautet (2007); Giménez *et al.* (2010).

1.2. Epidemiology

In the epidemiology field there are few studies regarding the incidence of mammary neoplasia in cats. In addition, researchers believe that the available data underestimate the true incidence of the disease, because most of the studies are archaic and outdate (Dorn *et al.*, 1968, MacVean *et al.*, 1978). According to Dorn *et al.* (1968), mammary tumors are the third most common tumor type in female cats (after skin tumors and lymphoma),

representing 12% of tumors in cats indepedent of sex. More recently, Vascellari and collegues (2009) reported that mammary tumors were the second most common tumor in cats and represent 16.3% of the tumors in cats independently of the sex (and 25.3% of the tumors in female cats).

Feline mammary tumors are almost exclusive of the female sex, such as human breast cancer (Sorenmo *et al.*, 2013). In humans, there are some risk factors well-established, like age, obesity, sedentary behaviour, alcohol consumption and smoking, shift work, hormonal or genetic status that may be implicated in mammary tumorigenesis (American Cancer Society, 2015).

In cats, only age, breed, and hormonal influence were already identified as risk factors for FMC. According to the literature, and as is described for women and bitch, feline mammary carcinoma incidence increases with age, and disease is predominantly seen in middle-aged to older cats. The mean age of diagnosis is between 10 to 12 years of age, with increasing risk up to 14 years of age, and carcinomas are more likely to occur in older cats, when compared with benign mammary tumors (Weijer & Hart, 1983; Sorenmo *et al.*, 2013; Zappulli *et al.*, 2015).

Based on the breed, two studies registered that Siamese cats appear to be overrepresented when compared to other breeds (Hayes *et al.*, 1981; Ito *et al.*, 1996). Finally, the exposure to ovarian hormones is also strongly implicated in mammary tumorigenesis in cat. Overley *et al.* (2005) concluded that sexually intact cats have a higher risk than spayed cats to develop mammary carcinomas (p=0.001, odd ratio [OR] 2.7) and also that the risk of the disease increase with age. Moreover, cats spayed prior to 6 months of age had a 91% reduction of the risk of develop mammary tumors (MT) and when the cat is spayed prior to one year the reduction of the risk lowed for 86%. Besides the ovarian hormones, there is also an increased risk of developing mammary cancer in queens subjected to regular administration of progestogens (Misdorp *et al.*, 1991). Finally, Overley and collegues (2005) concluded that parity did not affect mammary tumor development.

1.3. Dysplastic lesions and benign mammary tumors

Less than 15% of the feline mammary lesions are dysplastic lesions and benign neoplasias (Figure 2). The dysplastic lesions include ductal hyperplasia, lobular hyperplasia (epithelial hyperplasia, adenosis and fibroadenomatous change), cysts, duct ectasia and fibrosclerosis. Fibroadenomatous change (fibroepithelial hyperplasia, fibroepithelial hyperthrophy or feline mammary hyperthrophy) is a hormonal induced proliferation of interlobular ducts and periductal stromal cells (Misdorp *et al.*, 1999).

Benign tumors (Figure 2) in cat are uncommon and include adenoma (simple and complex), fibroadenoma, benign mixed tumor and duct papilloma (Misdorp *et al.*, 1999).

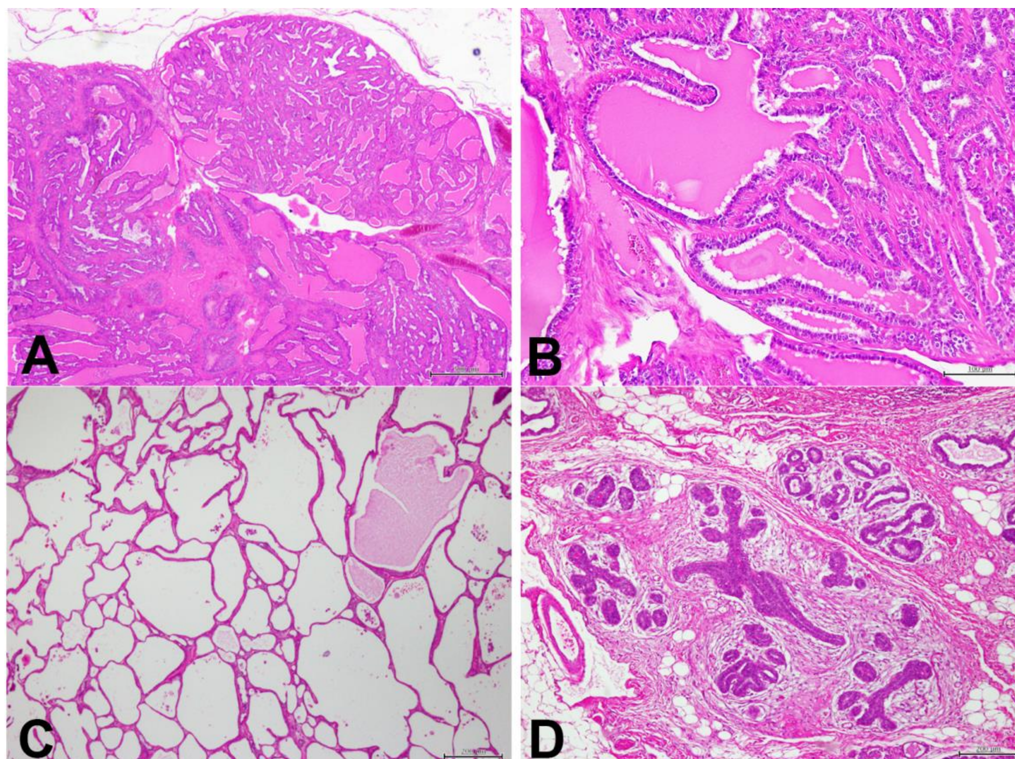

Figure 2. Dysplastic lesions and benign mammary tumors (Hematoxylin & Eosin [HE]).

A (20x) and **B** (100x) images represent a simple tubulopapillary adenoma in a female cat. The lesion is composed of a single layer of cells arranged in tubules that contain an amorphous amphophilic secretion. In **C** (40x) a feline mammary gland with duct ectasias is illustrated. Ectasias affect the tubules and also the acini. In the figure, dilated tubules that contain fluid are observed. **D** (40x) presents a fibroepithelial hyperplasia in a female cat. The lesions are clearly circumscribed, without being capsulated. There is a extremely marked proliferation of the intralobular duct system as well as of the intralobular connective tissue.

1.4. Malignant mammary tumors

Most of mammary tumors in cats are malignant (85% to 95%), and present an aggressive biological behaviour, with early lymphatic invasion and lymph node metastases. The malignant tumors types in cats are predominantly adenocarcinomas, specially the simple carcinomas like tubulopapillary and solid carcinomas (Figure 3A and 3B). Others types of mammary tumors include noninfiltrating (*in situ*) carcinoma, cribriform carcinoma (Figure 3C), invasive micropapillary carcinoma, squamous cell carcinoma, mucinous carcinoma (Figure 3D), 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). Carcinosarcomas, sarcomas and other non-epithelial neoplasias are very rare mammary tumors in cats (Misdorp *et al.*, 1999; Sorenmo *et al.*, 2013).

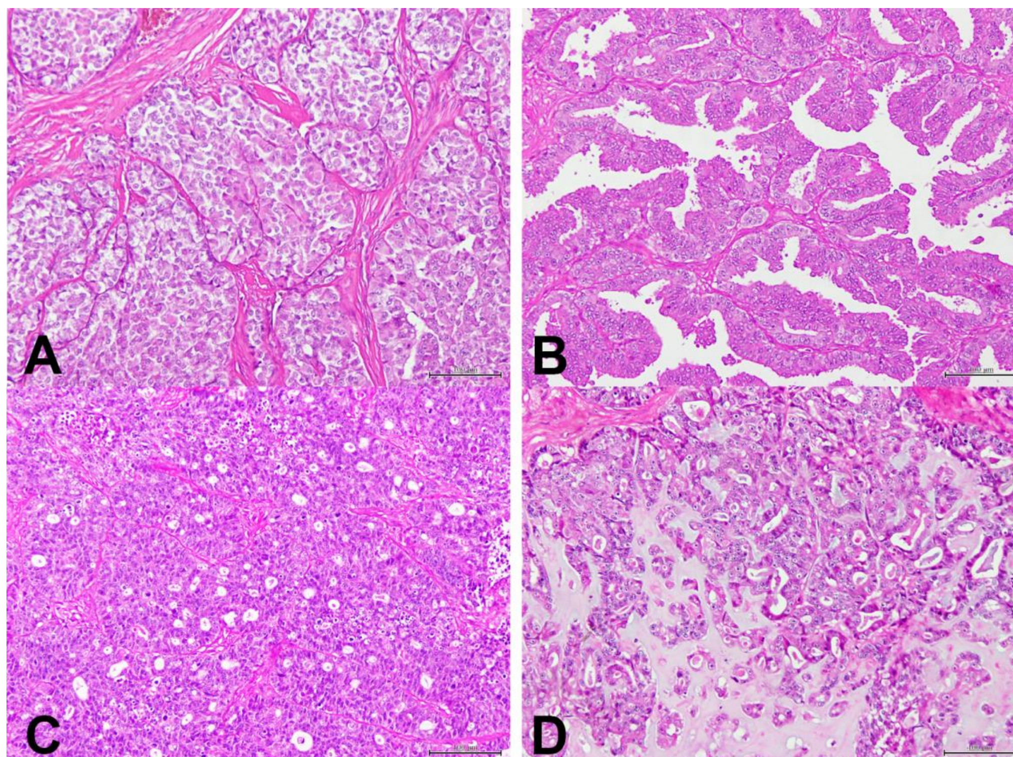

Figure 3. Feline malignant mammary tumors (100x, HE).

A, Solid mammary carcinoma of a female cat with 7,5 years old. It is evident that lobules are changed into solid tumor cell masses with reduced glandular structure. **B,** Tubulopapillary mammary carcinoma in a female cat. The tubules are predominantly arranged in a pedunculated papillary fashion. **C,** Cribriform mammary carcinoma. In the figure is evident the sievelike arrangement of the neoplastic epithelial cells, characteristic of this hystope. **D,** Mucinous mammary carcinoma in a female cat with 11 years old. It is an uncommon type of mammary carcinoma, characterized by the abundant mucin production.

1.4.1. Malignancy grade

In Human Medicine, Elston and Ellis method is the most common histological grading method for invasive carcinomas, and is correlated with the prognosis of the disease (Elston & Ellis, 1998).

This system, that was also adapted for feline mammary tumors, is based on the assessment of three morphological features: (1) the degree of glandular differentiation assessed by tubular formation; (2) nuclear pleomorphism and (3) mitotic activity (Table 1). Recently, another classification was suggested by Mills *et al.*, 2015, that consider lymphovascular invasion, nuclear form and mitotic counting (Table 2).

Notwithstanding, the two classification systems have already proven its prognostic value in cats with mammary carcinoma (Castagnaro *et al.*, 1998a; Seixas *et al.*, 2011; Mills *et al.*, 2015).

Table 1. Elston and Ellis grading system for evaluation of invasive mammary carcinoma in female cat.

Histologic feature	Score
Tubule formation	
tumor had more than 75% tubules	1
10-75% of tumor had tubule formation	2
<10% tubules	3
Nuclear pleomorphism	
small regular uniform cells	1
moderate nuclear size and variation	2
marked nuclear variation	3
Number of mitoses per 10 high power fields (HPF)	
0-5 mitoses per 10 HPF	1
6-10 mitoses per 10 HPF	2
>11 mitoses per 10 HPF	3

Point total	Grade
3-5	Grade I, well differentiated or low grade
6-7	Grade II, moderately differentiated or intermediate grade
8-9	Grade III, poorly differentiated or high grade

HPF, High power fields

Table 2. Mills *et al.* (2015) grading system for evaluation of invasive mammary carcinoma in female cat.

Histologic feature	Score
Lymphovascular invasion	
Absent	0
Present	1
Nuclear form	
≤5% abnormal	0
>5% abnormal	1
Mitotic count per 10 high power fields (HPF)	
≤62	0
>62	1

Point total	Grade
0	Grade I, low grade
1	Grade II, intermediate grade
2-3	Grade III, high grade

1.5. Clinical presentation

As indicated above (chapter 1.2), cats with mammary carcinomas are often old and may be sexually intact or spayed. Tumors are usually easy to detect on physical examination as they appear as firm and discrete masses in the mammary gland that can be detached or attached to the underlying tissue (Figure 4).

Multiple tumors are common (Figure 4A) and 60% of cats have more than one tumor at the time of diagnosis, according to Hayes & Mooney (1985). All glands are susceptible to tumor development although anterior glands are less commonly affected (Weijer & Hart, 1983). For this reason, a careful examination of the remaining mammary glands is always recommended in the clinical examination of the oncologic animal and for each mass the following features should be evaluated: size of the mass, consistency, mobility and the presence of skin ulceration (Weijer & Hart, 1983).

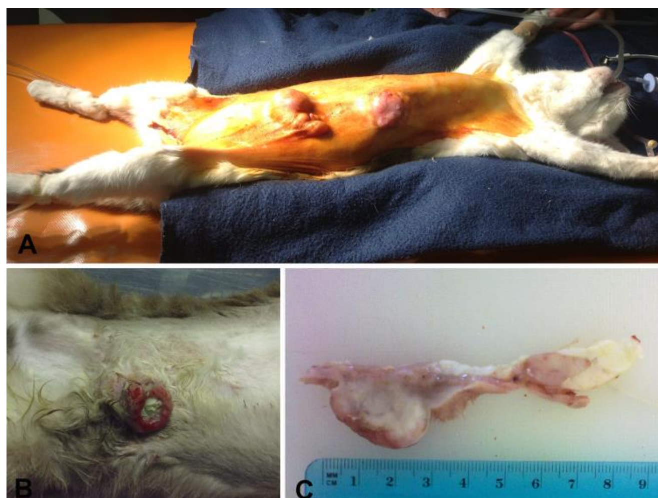

Figure 4. Clinical presentations for mammary tumors.

A, Multiple mammary tumors of a domestic shorthaired (DSH) female cat with 11 years. The masses were malignant (tubulopapillary carcinomas) and the animal also presented regional lymph node invasion by metastatic cancer cells (stage 3, T3, N1, M0). **B,** Siamese cat with 13 years old presenting a large ulcerated mammary tumor (stage 3, T3, N1, M0). **C,** Macroscopic examination of the mammary mass in picture B (sagittal cut, in the left), and a completely altered lymph node by metastatic lesions (sagittal cut, in the right).

Tumor size is a very important feature as it presents prognostic value and depends on how early the tumor is detected and how aggressive the tumor behaves. Usually, larger tumors may become ulcerated, inflamed and infected (Weijer & Hart, 1983; MacEwen *et al.*, 1984; Ito *et al.*, 1996).

FMC are typically very aggressive and present high capability to metastasize to regional lymph nodes and to distant organs such as lungs and pleura (Figure 5), which are the organs most commonly affected (Weijer & Hart, 1983). Moreover, authors report that 50% to 93% of cats with mammary carcinoma show metastasis at necropsy, making the inspection of the local lymph nodes in the clinical examination crucial (Hayden & Nielsen, 1971; Hahn *et al.*, 1994).

Considering the high incidence and the morbidity of FMC, a clinical and complete work-up is recommended and includes: a complete physical examination, a complete blood count, serum biochemical profile, serum T4 concentration, urinalysis, three-view thoracic radiographs (ventrodorsal, right and left lateral views), abdominal ultrasound or a computed tomography (CT) scan (Figure 6). All mammary masses and any palpable regional lymph node should be subjected to a fine-needle aspiration (FNA) or to a biopsy (Figure 7). Ulcerated lesions should be scraped and fluids from the affected glands should be examined, in order to achieve a diagnosis before surgery. All the mammary lesions and corresponding regional lymph nodes should be analyzed by histopathology.

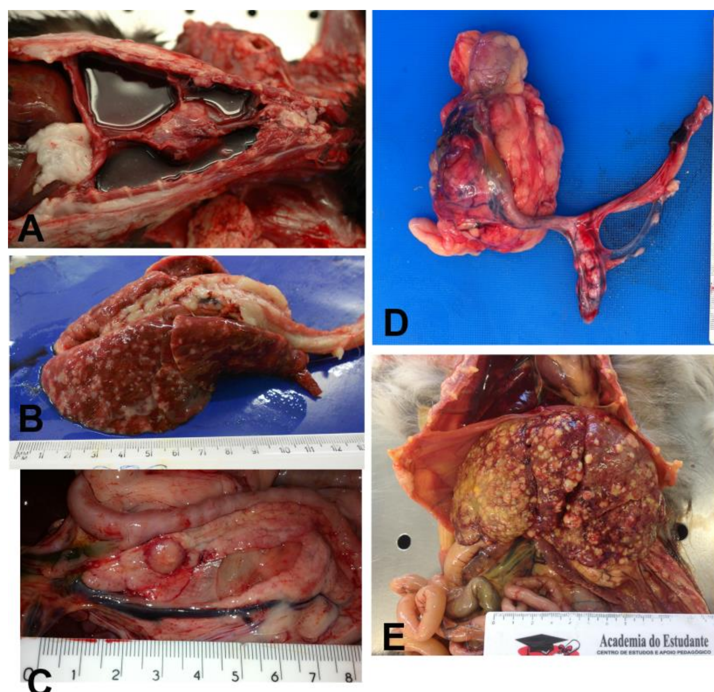

Figure 5. Metastatic lesions from mammary carcinomas in cats.

A, Necropsy of a 15 year old DSH female cat with pleural effusion and metastases in the pleura and **B,** lung. **C,** Necropsy of a 9 year old female cat with metatases in the pancreas, **D,** iliac lymph node and kidney, and other organs not represented in the picture (lung, liver and muscle). **E,** Necropsy of a Norwegian Forest Cat (NFC) 11 years old that presented liver metastases of a primary mucinous mammary carcinoma.

Similarly to humans and dogs, the mammary disease in cats is also staged, using a modification of the original system published by Owens (McNeill *et al.*, 2009), as is shown in Table 3. This staging system should not be used for mammary gland sarcomas.
Finally, inflammatory mammary carcinoma is a rare but a clinical important type of mammary tumor, which is also described in women and bitch. In these cases, the entire mammary chain or the mammary gland affected may appear edematous, swollen, warm and painful. The clinical identification of this type of carcinoma is very important once these animals are poor surgical candidates, due to the secondary postsurgical complications like nonhealing incisions, oedema and suture rejections (Pérez-Alenza *et al.*, 2004).

Table 3. Staging of Feline Mammary Tumors.

Stage	Tumor size	Lymphnode status	Metastasis
Stage 1	T1: <2cm	N0	M0
Stage 2	T2: 2-3cm	N0	M0
Stage 3	T1 or T2	N1 (positive)	M0
	T3: >3cm	N0 or N1	M0
Stage 4	Any	N0 or N1	M1

Legend: T, tumor; N, lymph node; M, metastasis; 0, absence of metastasis; 1, presence of metastasis.

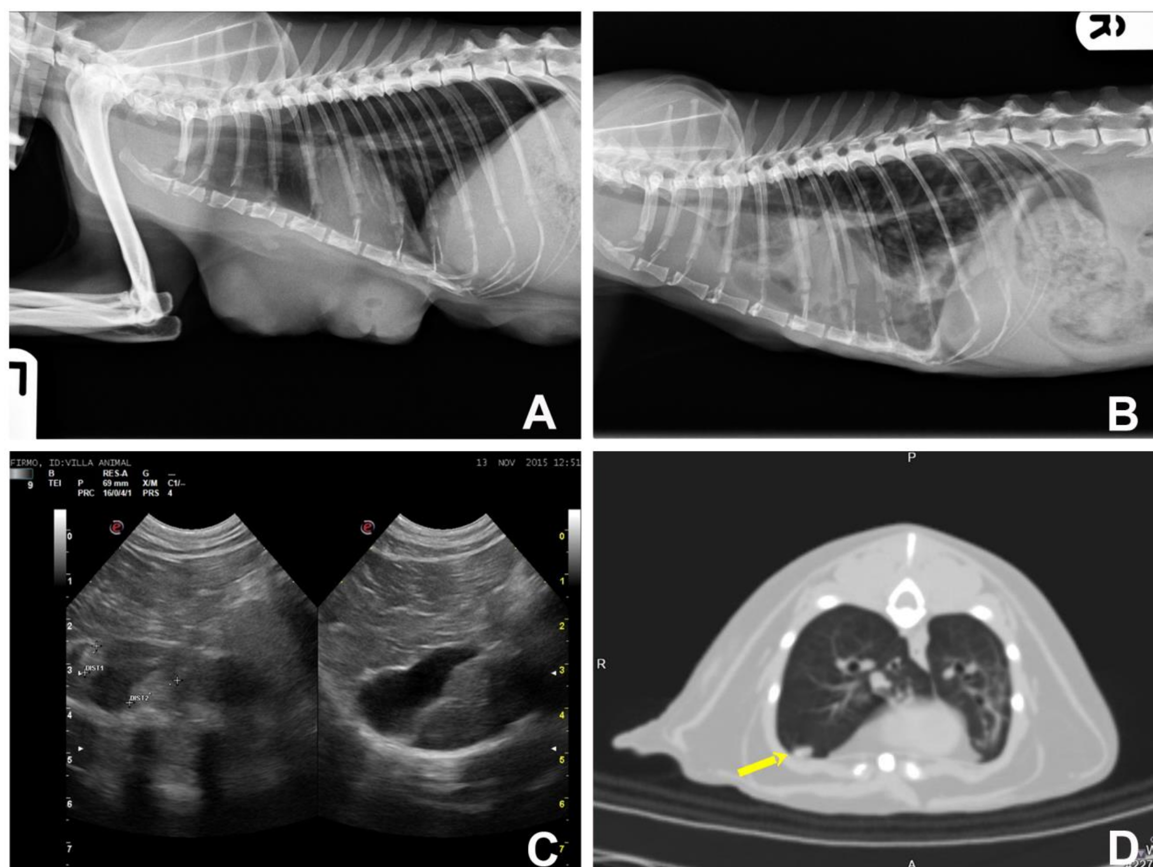

Figure 6. Imagiologic findings.

Left lateral **(A)** and right lateral **(B)** thoracic radiographs from a 14 year old queen with a solid mammary carcinoma before the surgery **(A)** and three-months after the surgery **(B)**. **A,** Radiograph shows masses in the thoracic mammary region. **B,** In these radiograph, it is visible an extensive pleural effusion and numerous ill-defined round soft tissue opacities that are scattered through the lung fields consistent with pulmonary metastasis. **C,** Abdominal ultrasound in a cat with a tubulopapillary mammary carcinoma. There are two ill-defined rouded hyperechoic lesions in the hepatic parenquima, consistent with hepatic metastasis. **D,** CT transverse image of a cat with tubular mammary carcinoma. In the CT is visible a soft tissue nodule in the pulmonary parenchyma (yellow arrow), consistent with pulmonary metastasis, which was subsequently confirmed at necropsy.

1.6. Treatment options

Surgery is traditionally the required treatment for FMC and the surgical extension has already been associated to the disease free survival (DFS) period (MacEwen *et al.*, 1984). Cats with mammary lesions are usually subjected to a simple lumpectomy, mastectomy, regional mastectomy, or a chain mastectomy that can be unilateral or bilateral. The goal of surgical intervention is to remove the current tumor or tumors with clean margins, in order to prevent the emergence of new tumors in the remaining mammary glands.

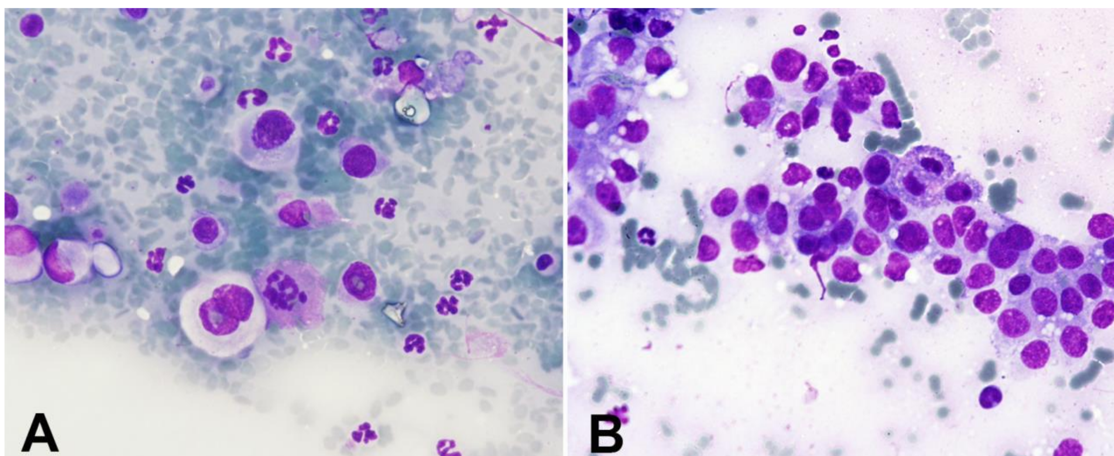

Figure 7. Fine-needle aspirate of a solid mammary carcinoma (Giemsa, 40x).

A, The cytological smear displayed multinucleated tumor cells and pleomorphic tumor cells, with marked anisocytosis, anisokaryosis and multiple preominent nucleoli suggesting a high level of tumor aggressiveness. Mitoses are also present. **B,** Presence of a homogeneous population of epithelial cells with high nuclear:citoplasmatic ratios, pleomorphic cells and the presence of mitoses.

In cats, radical mastectomy (unilateral for cats possessing a single tumor or a 2-staged bilateral chain mastectomy for animals with bilateral tumors) results in a significant larger DFS when compared with cats receiving a conservative chain mastectomy (MacEwen *et al.*, 1984). Thus, unilateral or staged bilateral mastectomy is the recommended treatment for FMC. In some cases a bilateral mastectomy could be performed in a single surgery, if the postsurgical tension is minimal (when the animal presents excessive mammary or adipose tissue, for example). For tumors that are fixed, muscular fascia or portions of the body wall should also be resected (Sorenmo *et al.*, 2013).

Aggressive assessment of the regional lymph node is justified by the high metastatic potential of FMC and by the poor prognosis that is associated to the presence of lymph node metastasis. This could be assessed with an ultrasound-guide FNA or through the histopathological evaluation of the resected regional lymph node during surgery (Sorenmo *et al.*, 2013).

Early detection and aggressive surgery (including prophylactic chain mastectomy) can result in long-term survival in cats with early stage mammary tumors. However, cats with late diagnosis or later stages of the disease are not treated effectively with surgery alone. In those cases the use of adjuvant doxorubicin-based chemotherapy should be considered (Novosad *et al.*, 2006; McNeill *et al.*, 2009). In one multi institutional retrospective study, a comparison between cats with mammary carcinomas receiving surgery *versus* cats receiving surgery with adjuvant chemotherapy demonstrated that the subset of cats having unilateral chain mastectomy followed by chemotherapy presented an longer overall survival (OS) than the other group of animals (McNeill *et al.*, 2009).

Alternative chemotherapy protocols include a combination of doxorubicin and cyclophosphamide or doxorubicin-based chemotherapy and a nonsteroidal anti-inflammatory drug (meloxicam). However, survival studies based on these protocols have not been performed, so their efficiency cannot be determined in cats with mammary tumors (Mauldin *et al.*, 1988; Sorenmo *et al.*, 2013; Borrego *et al.*, 2009).

Concerning others treatments, as hormonal therapy, they are unlike to be effective once studies indicate that FMC presents a low expression of hormonal receptors (de las Mulas *et al.*, 2002; Millanta *et al.*, 2005b; Millanta *et al.*, 2006a; Burrai *et al.*, 2010) and studies that evaluate this therapy in cats with FMC were not found (Sorenmo *et al.*, 2013).

Recently, promising results were obtained using oncolytic virotherapy using FMC cell lines (Adelfinger *et al.*, 2014). This therapy is based on the capacity of oncolytic viruses infecting and promoting cancer cells lysis without extensively damaging the surrounding normal tissue.

Finally, and despite the high incidence of distant metastasis in these animals, there are almost no recent studies to search for effective adjuvant systemic treatments for FMC.

1.7. Prognostic factors

Prognosis is generally assessed using the one-year postsurgical rate of survival/remission in cats, which is comparable to the ten-year postsurgical survival/remission rate generally used in humans. DFS and postsurgical remission rate at a fixed interval are considered better prognostic indicators, at least in cats, since the overall survival time might be influenced by other factors such as concomitant diseases or euthanasia (MacEwen *et al.*, 1984; Matos *et al.*, 2012).

There are some studies related to survival in cats with feline mammary carcinomas. For example, a review by Zappulli *et al.* (2015) indicated that survival time after primary tumor detection rounds the 12 months in cats depending on the clinical staging and on the tumor excision. Additionally, several parameters are considered important prognostic indicators for FMC such as age, size of the primary tumor, malignancy grade, lymph node involvement, number of mitoses, extent of necrosis, completeness of surgical resection and type of invasion (MacEwen *et al.*, 1984; Ito *et al.*, 1996; Sarli *et al.*, 2003).

1.7.1. Clinical features

Usually, age is an associated indicator since old animals show lower survival rates (Weijer & Hart, 1983; Ito *et al.*, 1996). More recently, another study correlate cats with old age with a shorter DFS and OS but the prognostic significance was lost when multivariate analysis was performed (Seixas *et al.*, 2011).

Clinical staging of FMC, based on the TNM classification of malignant tumors system, proved to be an important prognostic factor. According to Hughes & Dobson (2012) the cats with

more advanced clinical stages (stage III and IV) presented a lower OS, when comparing to animals with earlier clinical stages (stage I and II).

Consequently, the features that constitute the TNM system (size, lymph node status and distant metastasis) also exhibit prognostic value, when were considered individually (Seixas *et al.*, 2011; review in Zappulli *et al.*, 2015).

Seixas *et al.* (2011) proved that lymph node metastasis was an independent prognostic factor for OS, with female cats that presented lymph node metastasis being associated with shorter OS. Still, the study did not reach to a significant value in the DFS analysis.

Two studies pointed that surgical procedure is a potential prognostic factor in FMC, once cats with nonmetastatic mammary carcinomas treated with radical mastectomy had longer DFS and OS when compared to those that received a more conservative surgery (Weijer & Hart, 1983; MacEwen *et al.*, 1984).

1.7.2. Histologic features

One of the most studied parameters in FMC is tumor size, which has already proved to have prognostic value (Weijer & Hart, 1983; MacEwen *et al.*, 1984; Ito *et al.*, 1996; Seixas *et al.*, 2011). Cats with larger tumors were consistently associated to lower OS and to lower DFS, independently when considered the tumor volume or the tumor diameter.

There are several studies related to the tumor histotype though. A number of authors could not reach to any prognostic value (Castagnaro *et al.*, 1998a; Misdorp *et al.*, 1999; Millanta *et al.*, 2002a). However, Seixas *et al.* (2007) demonstrated that some histotypes were associated with OS and DFS. Cats with mammary invasive micropapillary carcinoma presented a significantly shorter OS and DFS when compared to cats with other FMC. In another study, these authors (Seixas *et al.*, 2008) also evidenced that complex feline mammary carcinomas were significantly less aggressive than simple tumors, once they presented a larger OS. Finally, cats with tubulopapillary and complex mammary carcinomas had demonstrated a significantly longer OS when compared to cats with solid and invasive micropapillary mammary carcinomas (Seixas *et al.*, 2011).

As for humans and dogs, malignancy grade was associated with the cat outcome (Karayannopoulou *et al.*, 2005; review in Rakha *et al.*, 2010), presenting prognostic value in several studies. Cats with undifferentiated tumors (Grade III) present shorter OS and DFS when compared to animals with grade I and II tumors (Castagnaro *et al.*, 1998a; Millanta *et al.*, 2002a; Seixas *et al.*, 2011). Recently, Mills *et al.* (2015) proposed a novel classification system that includes lymphovascular invasion, which has also demonstrated its prognostic value in cats.

According to Weijer & Hart (1983) there are several histological features associated with OS and DFS in cats, like nuclear and cellular atypia, necrosis, chronic inflammation around the tumor, lymphatic emboli, infiltrative growth, ulceration and mitotic count.

1.7.3. Immunohistochemical features

1.7.3.1. Hormone receptors (HRs)

Despite estrogen receptor (ER) and progesterone receptor (PR) expressions being implicated in the initial stages of mammary tumor development, some studies point out that most of feline mammary carcinomas are ER and PR negative (de las Mulas *et al.*, 2002; Millanta *et al.*, 2005b; Millanta *et al.*, 2006a; Burrai *et al.*, 2010). About one-third of FMC are PR positive, as concluded by de las Mulas and collegueas (2002). This low HRs positivity is consistent with the higher rate of malignancy and more aggressive tumor behaviour. Nevertheless, there were no studies found that correlated ER or PR with survival rates.

Conversely, normal mammary tissue and dysplastic lesions in mammary gland usually express ER and PR (Millanta *et al.*, 2005b; Millanta *et al.*, 2006a; Burrai *et al.*, 2010). Fibroepithelial hyperplasia, a progesterone-induced change, has been reported to have high PR expression and can be effectively treated with ovariohisterectomy or antiprogesterones therapies (Sorenmo *et al.*, 2013).

1.7.3.2. Human epidermal growth factor receptor-2 (HER2)

There are several studies (De Maria *et al.*, 2005; Millanta *et al.*, 2005a; Winston *et al.*, 2005; Ordás *et al.*, 2007; Burrai *et al.*, 2010; Rasotto *et al.*, 2011; Santos *et al.*, 2012) related to the feline homologue of HER2 (*f*HER2) but only one correlated the overexpression of *f*HER2 with a shorter OS, in a two-year follow-up study (Millanta *et al.*, 2005a).

1.7.3.3. Proliferative markers

The use of some selected proliferative indices, such as AgNOR (Argyrophilic nucleolar organiser regions) protein quantification, mitotic index and immunohistochemical detection of cell cycle associated antigens (Proliferating cell nuclear antigen [PCNA], Ki-67), has been proposed for diagnostic and prognostic purposes.

The mitotic index was significantly associated with OS and DFS in some studies (Weijer & Hart, 1983; Sarli *et al.*, 2003; Seixas *et al.*, 2011), and cats with mammary carcinomas presenting a low mitotic index were associated with larger survival times and DFS, when compared with animals with high mitotic rates.

In relation to Ki-67 index value, a correlation with increased survival rate in one year postsurgery was found in cats with lower levels for this indicator (Castagnaro *et al.*, 1998c) and similar results were obtained by other authors (Preziosi *et al.*, 2002; Millanta et al 2006a; Seixas *et al.*, 2011).

Cats with lower AgNORs counts (<5.9 AgNORs per cell) also presented longer survival times (Castagnaro et al 1998b).

1.7.3.4. Other biomarkers

Recently, several biomarkers were explored in order to evaluate their prognostic value in FMC.

The role of cyclooxygenase-2 (COX-2) as an indicator of aggressiveness and prognosis in FMC has been investigated, especially because it could be predictive for anti-inflammatory therapies (Millanta *et al.*, 2006b; Sayasith *et al.*, 2009). One of these studies has already associated high COX-2 expression to shorter OS (Millanta *et al.*, 2006b).

The vascular endothelial growth factor (VEGF) expression was directly associated with survival, and cats with mammary carcinomas with VEGF overexpression presented shorter overall survival times (Millanta *et al.*, 2002b).

Phosphatase and tensin homolog (PTEN) and phosphorylated protein kinase B (p-AKT) are examples of other proteins with recognized importance in human breast cancer and that have already been subjected to investigation in FMC but have failed to be correlated with the prognosis (Ressel et al 2009; Maniscalco *et al.*, 2012).

1.8. Progression of the disease

It is not evident how cancer cells acquire metastatic capability. According to the latest researches, there are two major models: the progression model and the early oncogenic model (Fidler & Kripke, 1977; Fidler, 2003; Ramaswamy *et al.*, 2003; Yang *et al.*, 2014). In the progression model, the metastatic phenotype is acquired in a small fraction of cells within the heterogeneous primary tumour, whereas the early oncogenic model suggests that the metastatic potential (the genetic and epigenetic alterations) emerge at the same time as the events that contribute to the initial primary tumour development (Fidler & Kripke, 1977; Ramaswamy *et al.*, 2003).

In feline mammary tumors, rates of metastases have been reported to range from 50 to 90%. Frequently, these affect the regional lymph nodes (83%), lungs (83%), liver (25%) and pleura (22%) (reviewed in Zappulli *et al.*, 2015). Unfortunately, no studies could be found in the scientific literature about biological information related to the metastatic lesions of FMC and the clinical management of the cats with metastatic disease.

2. HER2 or HER2/neu or c-erbB-2

The HER2 oncogene, also known as ERBB2 or HER-2/neu or c-ErbB-2 proto-oncogene (Coussens *et al.*, 1985; Semba *et al.*, 1985), is the human homologue of the *neu* oncogene identified in DNA samples obtained from rat neuro/glioblastomas induced by ethylnitrosourea (Shih *et al.*, 1981; Padhy *et al.*, 1982). These are also homologous of the v-erbB (avian erythroblastosis virus) viral oncogene that induces sarcomas and erythroblastosis in infected birds (Sergeant *et al.*, 1982; Jansson *et al.*, 1983; Spurr *et al.*, 1984; King *et al.*, 1985).

The HER2 oncogene is located on the long arm of chromosome 17, at 17q21 and comprises 27 coding exons. It encodes a 185 kDa transmembrane tyrosine kinase receptor with homology with the epidermal growth factor receptor (EGFR or HER1) and belonging to the epidermal growth receptor (EGR) family (Akiyama *et al.*, 1986; Rajkumar & Gullick, 1994; Rampaul *et al.*, 2002). This family consists in four homologous receptors (HER1 or EGFR, HER2, HER3 and HER4) and 10 genes that code for ligands. Excluding HER2, all these receptors are activated upon the interaction and binding to extracellular ligands. HER2 is an orphan receptor, whereby its activation occurs through homodimerization (HER2 and HER2) or heterodimerization with other receptors of the HER family (Rampaul *et al.*, 2002). This process triggers intrinsic tyrosine kinases (TK) activity of the receptors through the phosphorylation of specific TK residues, located in the intracellular domain of the receptor. The phosphorylation sites act as docking stations for signalling molecules involved in the regulation of intracellular signalling cascades (Park *et al.*, 2008). Thus, these proteins are tyrosine kinases and are transmembrane glycoproteins, composed by an N-terminal extracellular domain (ECD), a single transmembrane helix (the transmembrane domain), and an intracellular domain (ICD). The ICD is composed by a TK domain and an intracellular regulatory domain with the exception of HER3 that has impaired kinase activity (Kraus *et al.*, 1989; Plowman *et al.*, 1993; Guy *et al.*, 1994; Yarden, 2001).

The extracellular domain of HER2 is approximately 600 residues long and is constituted by four domains (Figure 8): two leucine-rich domains (I/L1 and III/L2), responsible for the ligand binding to the other HER proteins, and two cysteine-rich domains (II/CR1 and IV/CR2), with disulphide-bond connectivities responsible for ligand-induced dimerization. Alternative splicing of HER2 can also generate a truncated product that is constituted by the HER2 ECD rather than the full-length protein (Scott *et al.*, 1993).

The transmembrane domain presents a single hydrophobic helix and is the site of the mutation in the original rodent *neu* oncogene. This mutation consists in a missense mutation of residue 664, resulting in a valine to glutamic acid substitution which is responsible for the tumorigenicity of the *neu* oncogene (Bargmann & Weinberg, 1988b).

Finally, the intracellular domain, also designated by cytoplasmic region, is approximately 500 residues long and presents a highly conserved tyrosine kinase domain and a regulatory C-

terminal trail (Kraus *et al.*, 1989; Plowman *et al.*, 1993; Guy *et al.*, 1994). The TK domain includes the enzymatic sequences capable of TK activity (Yarden, 2001).

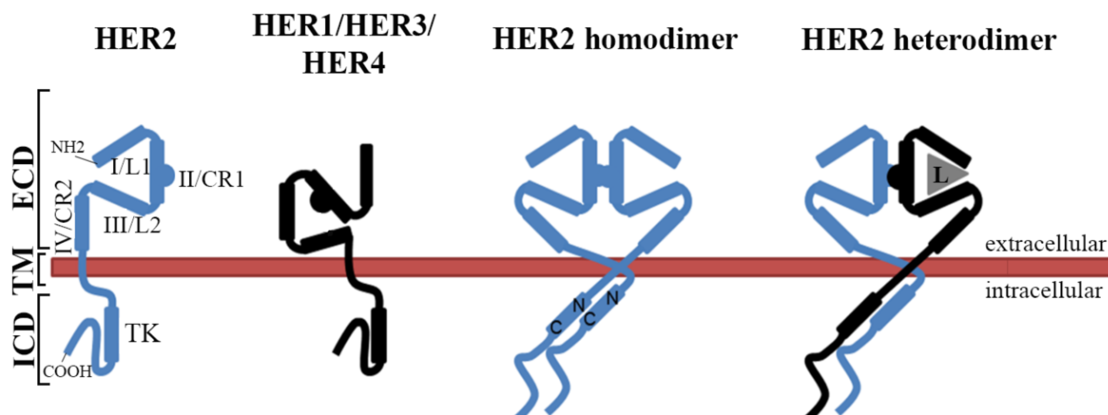

Figure 8. Conformation of HER proteins and homo and heterodimerization.

While the extracellular domain (ECD) of HER2 constitutively adopts an open conformation, HER1, HER3 and HER4 depends on ligand (L) binding to change from a closed conformation to an open one, and thus being able to participate in dimerization. When dimerization occurs, the TK domains interact in an asymmetric manner where C-terminal of one TK relates with the N-terminal of the other receptor, activating the signalling pathways. I/L1, II/CR1, III/L2, IV/CR2 corresponds to the four domains that constitute the ECD. ECD, Extracellular domain; TM, Transmembrane domain; ICD Intracellular domain; TK, tyrosine kinase; L, ligand. *Adapted from* Bertelseng & Stang (2014)

2.1. Function

Expression patterns of HER receptors and their ligands are important in epithelial, mesenchymal and neuronal cells. They play essential roles in the development of cells into different organs, especially in the mid-gestation processes, like the development of the cardiovascular system, nervous system and mammary gland (Ménard *et al.*, 2004). Therefore, normal ovaries and kidney present HER2 expression at low levels, as well as epitelial cells of gastrointestinal, respiratory, reproductive and urinary tract, skin, breast and placenta (Press *et al.*, 1990; Rampaul *et al.*, 2002).

HER2 deficiency appears to be specially related to aberrant cardiac and peripheral nervous system development (Lee *et al.*, 1995). HER2 knockout animals present a failure in the development of trabeculae fingerlike extensions of the ventricular myocardium. Consequently, this deficiency results in an enlarged common ventricle, a reduced blood flow and an irregular cardiac beat, which leads to the development of a severe dilated cardiomyopathy by the second postnatal month and to the dead of the animal (Meyer & Birchmeyer, 1995; Ozcelik *et al.*, 2002; Ménard *et al.*, 2004). Moreover, in adults, HER2 receptor is expressed mainly in T-tubules of the cardiomyocytes and is considered crucial for heart function (Ménard et al 2004). Garratt *et al.* (2003) demonstrated that loss of HER2 in adult mice can cause dilated cardiomyopathy, a relevant information for the management of

the patients under the administration of specific therapies with anti-HER2 monoclonal antibodies (Ozcelik *et al.*, 2002; Negro *et al.*, 2004; Pugatsch *et al.*, 2006).

In vivo studies about the nervous system development, HER2 was the first signalling molecule to demonstrate a role in schwann cell myelination and in oligodendrocyte differentiation (Kim *et al.*, 2003; Ménard *et al.* 2004).

In mammary gland, the epidermal growth receptor family plays several roles in the regulation of the growth, differentiation, apoptosis and remodulation of the normal mammary tissue. In the various stages of the mammary gland development, the receptors are differentially expressed, with HER2 and EGFR presence in all of the major cell types at the initial development. Nevertheless, they are preferential expressed in the lactating ducts and alveoli of the developed gland and constitute the principal components of EGR family in the nulliparous gland (Schroeder & Lee, 1998). In puberty, EGFR and HER2 are highly expressed in mammary glands and this expression decreases in the late-stage of pregnancy and during lactation (Sebastian *et al.*, 1998). Also, HER3 and HER4 are preferential expressed in the alveoli (Schroeder & Lee, 1998). Therefore, the absence of HER2 does not affect animal's ability to lactate, contrasting with HER3 and HER4 that are overexpressed in mammary glands mostly during pregnancy and lactation (Ménard *et al.*, 2004).

2.2. HER network

HER2 and the others members of the EGR family share a complex biological network (Figure 9) composed by an input layer with the membrane receptors and their ligands that triggers the signal from the extracellular medium; the signal-processing layer that corresponds to where the tyrosine kinase is phosphorylated and thus activates the signalling pathways; and the output layer, where occurs the activation of transcription factors that regulate genes evolved in several cellular functions like cell division, migration, adhesion, differentiation and apoptosis (Yarden & Sliwkowski, 2001; Benusiglio, 2007).

The four members of this family (HER1, HER2, HER3 and HER4) are activated upon ligand-induced receptor homo and heterodimerization (Figure 8), with exception of HER2. In case of HER2, it does not bind with any of the known ligands with high affinity (Pinkas-Kramarski *et al.*, 1996; Marmor *et al.*, 2004). Nevertheless, HER2 is the preferred heterodimeric partner for the other HER receptors, once it exists in a dimerization-favourable conformation on the cell surface. In addition, it binds to a much larger subset of phosphotyrosine-binding protein when compared to the other receptors (Pinkas-Kramarski *et al.*, 1996; Tzahar *et al.*, 1996; Graus-Porta *et al.*, 1997; Marmor *et al.*, 2004; Citri & Yarden, 2006). Crystallographic data of HER2 shows that this receptor is permanently in an open configuration, i.e. the domain II is always ready to dimerize allowing the ligand-bound conformation with the other HER receptors (Garret *et al.*, 2003). Conversely, the other HER receptors are dependent of a ligand that is capable to induce a conformational change to an open configuration, enabling

the domain II of the ECD to mediate receptor-receptor interactions as illustrated in Figure 8 (Park *et al.*, 2008).

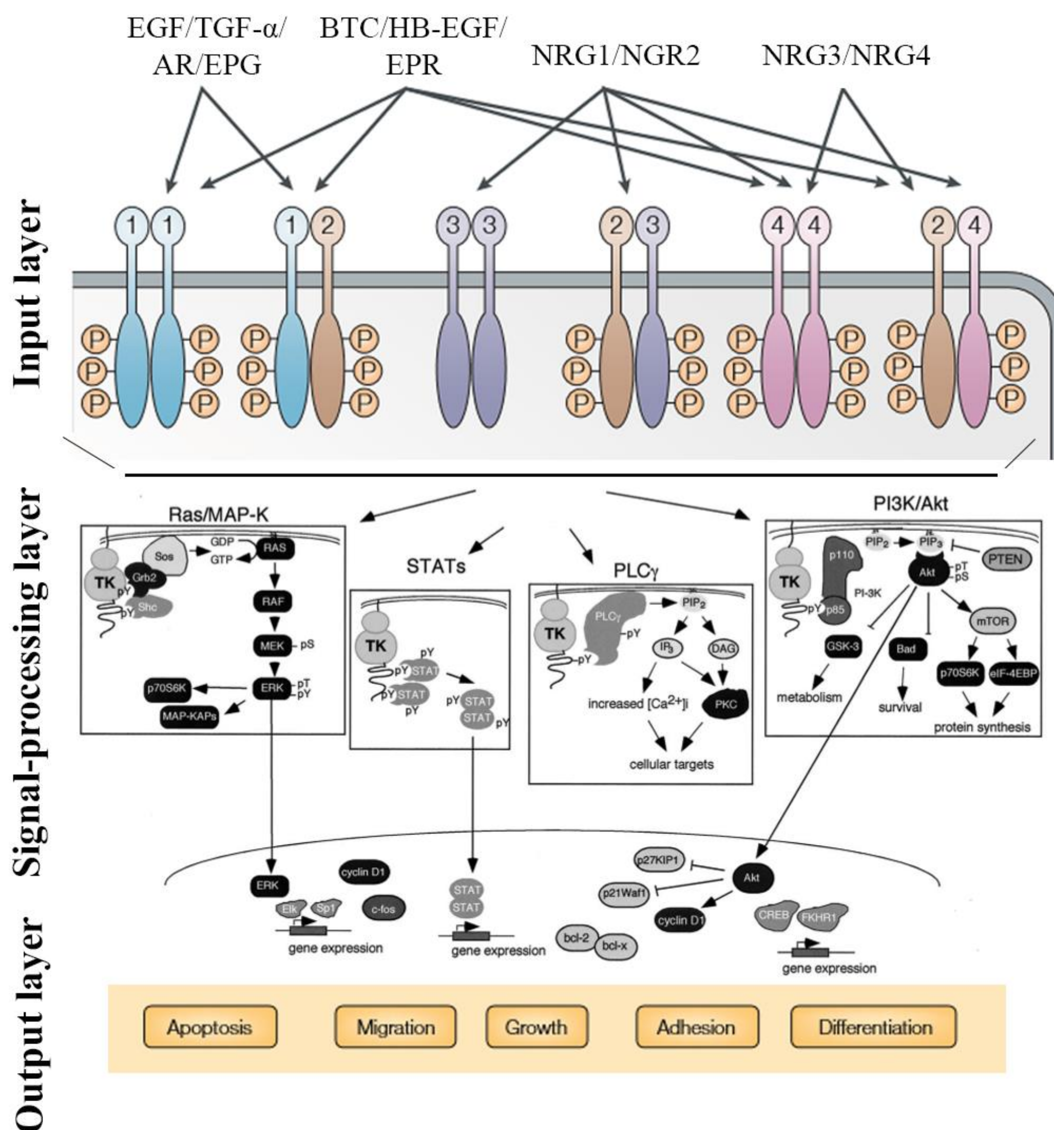

Figure 9. The HER signalling network.

Ligands and the ten dimeric receptor combinations comprised the **input layer**. Ligands and their receptor specificity are represented. HER2 does not binds with any ligand but is the preferred dimerization partner for all the other EGF receptors. HER3 homodimer is catalytically inactive and only acquires signalling potential when it dimerizes with another EGF receptor. In the **signal-processing layer** there is an activation of various signalling pathways through the phosphorylation of specific tyrosine kinases residues, due to the dimerization of the receptors. The figure represents the most importants pathways that are activated through HER dimerization, namely the Ras-MAPK, PI3K-Akt (or PI3L-PKB/Akt), PLC-PKC, and the STAT pathways. Collectively, these pathways culminate in the **output layer**, where transcription factors that regulate genes involved in cellular apoptosis, migration, growth, adhesion and differentiation are activated. *Adapted from* Marmor *et al.* (2004); Hynes & Lane (2005); Yarden & Sliwkowski (2001)

The outcome of the receptors activation depends on the composition of the receptor pair and the identity of the ligands involved, which determine the signalling pathways induced as well as their magnitude and duration (Marmor *et al.*, 2004). Besides the 4 receptors, there are several ligands that are involved such as epidermal growth factor (EGF), transforming growth factor-α (TGF-α), amphiregulin (AR), epigen (EPG), epiregulin (EPR), betacellulin (BTC), heparin-binding EGF-like growth factor (HB-EGF) and neuregulins (NRG) 1 to 4, illustrated in Figure 9 (Mukohara, 2011; Arteaga & Engelman, 2014). These ligands can be divided in three groups: 1) EGF, TGF-α, AR and EPG that specifically binds with EGFR; 2) BTC, HB-EGF and EPR that binds with EGFR and HER4; 3) NRG1 to NRG4, of which NRG1, NRG2 bind with HER3 and HER4 and NRG3 and NRG4 are only associate with HER4 (Hynes & MacDonald, 2009).

In the resting state, the receptors exist as monomers. As a result of activation by ligand-induced dimerization in the extracellular domain, they trigger the cytoplasmic kinase domain that phosphorylates tyrosines in the C-terminal tail region of each receptor, in the intracellular domain (Hynes & MacDonald, 2009). Phosphorylation triggers the association of specific signalling molecules that initiate downstream signalling events (Figure 9). In summary, HER receptors possess multiple tyrosine phosphorylation sites that will induce specific signalling pathways (Hynes & Lane, 2005; Citri & Yarden, 2006). Note that, despite HER3 has impaired kinase activity, tyrosine phosphorylation occurs as long as HER3 dimerizes with another receptor (HER1, HER2 and HER4) being the preferential partner for HER2 dimerization (Thazar *et al.*, 1996).

Figure 9 also represents the three best characterized signalling pathways that are Ras-mitogen-activated protein kinase (Ras-MAPK), phosphatidylinositol 3 kinase-protein kinase B (PI3K-PKB/Akt) and the phospholipase C-protein kinase C (PLC-PKC) pathway (Marmor *et al.*, 2004). The transcription factors activated by these pathways regulate genes involved in several cellular processes, such as migration, cell division, adhesion, angiogenesis, differentiation and apoptosis (Marmor *et al.*, 2004; Hynes & Lane, 2005; Citri & Yarden, 2006).

To control the amplitude, kinetics and frequency of the output signals of the HER network, several regulation systems are involved, as the positive-feedback loops, the negative regulatory pathways and the buffering systems. These processes are described below.

Positive-feedback loops are similar to the enzymatic networks in which positive-feedback loops enhance the amplitude and prolong the active state of signalling pathways. In the case of HER network, HER2 can be considered as an important positive regulator, because it is the preferred receptor and HER2 heterodimers have a higher ability to evade negative regulation. Another important mechanism of positive feedback is based on autocrine and

paracrine loops, with EGF-like ligands, as well as angiogenic factors, which are produced following receptor activation (Citri & Yarden, 2006).

Negative regulatory pathways include the downregulation of HER receptors by ligand-induced receptor endocytosis and the inhibition of HER2 activity by an alternative HER2 product, herstatin (Azios *et al.*, 2001; Wang *et al.*, 2004; Citri & Yarden, 2006):
Negative regulatory pathways can be induced through EGFR family into which a vast cellular effort is invested (Marmor *et al.*, 2004).
Multiple molecular mechanisms, including post-translational modifications, compartmentalization or catalytic inactivation, participate in signal attenuation. It is important to distinguish between general attenuation that occurs at the level of the ligand–receptor complex, and pathway-specific inactivation. Negative regulators can pre-exist or be synthesized following stimulation of the ligand-receptor complex. The pre-existing negative regulators usually control the receptor dephosphorylation and degradation. Thus, signal attenuation can be induced by dephosphorylation of the EGF receptors, using several phosphatases, such as density-enhanced phosphatase-1 (DEP-1) or protein tyrosine phosphatase-1B (PTP1B) (Citri & Yarden, 2006).
Based on the newly synthesized negative regulators, their levels of expression rise as a result of the activation of the signalling pathways. In addition, they are crucial for HER homeostasis, promoting the degradation of the receptors and ensuring the appropriate tuning of signals both during development and in adult tissues (Marmor *et al.*, 2004; Citri & Yarden, 2006).
In the negative regulatory pathways, one of the most effectives systems that attenuate the network signalling is the receptor internalization, especially if it is coupled to degradation of the receptors in lysosomes (Wiley, 2003). Receptor dimers can be internalized through clathrin-dependent or clathrin independent regions of the plasma membrane (Figure 10), forming the endocytic vesicles, which could promote the receptor recycling back to the cell surface, the degradation of the receptor, through lisosome activity or the translocation into the nucleus, as discussed below (Waterman & Yarden, 2001; Wiley, 2003; Wang *et al.*, 2010).

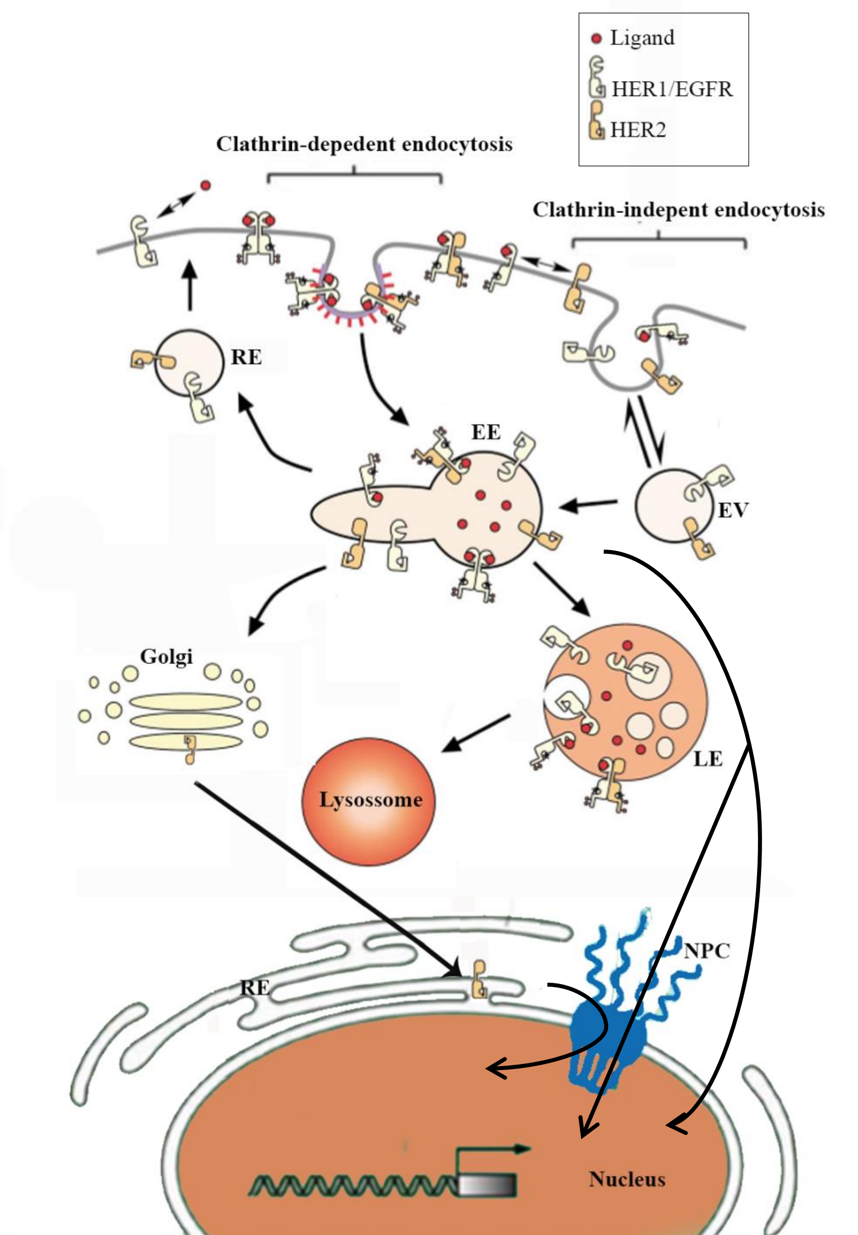

Figure 10. Trafficking of EGFR family.

Only the EGFR and HER2 proteins are shown, but the behaviour of the others EGF receptors (HER3 and HER4) are similar. The endocytic vesicles (EV) carrying EGF receptors from either clathrin-dependent or clathrin-independent endocytosis subsequently fuse with the early endosomes (EE). After this, the EGF receptors can be subjected to three events: EGFR and HER2 can be recycled back to the cell membrane; these receptors can be degraded, by the fusion of the late endosomes (LE) with lysosomes; the internalized EGFR and HER2 can also be transported to the nucleus from early endosomes to the endoplasmatic reticulum (RE) and then nuclear transport occurs through the nuclear pore complex (NPC). The receptors can also pass from the EE to the nucleus by retrograde transport from Golgi complex to RE and then into the nucleus. The transport through the recycling endosomes or the late endosomes has not been studied yet. *Adapted from* Wiley (2003); Wang *et al.* (2010).

In addition, alternative splice variants of HER can inhibit the receptor's signalling, such as herstatin and p100. Herstatin is a splicing variant of HER2 that is generated by retention of the intron 8 in HER2 alternative mRNA, and is composed by an extracellular domain and a novel C-terminus. This soluble form can interact with the full-length receptor and thus inhibit its activity and dimerization (Azios *et al.*, 2001; Koletsa *et al.*, 2008; Wang *et al.*, 2013a).
The p100 is another soluble form of HER2 that interferes with the oncogenic activity of wild-type HER2 (Jackson *et al.*, 2013). It was associated with inhibition of tumor cell proliferation and with the decrease of signalling induction of MAPKpathways (Scott *et al.*, 1993; Jackson *et al.*, 2013). The p100 protein is composed by the extracellular domain of the full-length protein HER2 and results of the intron 15 retention (Jackson *et al.*, 2013; Wang *et al.*, 2013a).
More recently, other alternative splice variants of HER2 have been unravelled, like Δ16HER2 and the carboxyl-terminal fragment (CTF) 611-CTF, 687-CTF and 648-CTF, as further described in chapter 2.3 (Wang *et al.*, 2013a). Contrasting with herstatin and p100, Δ16HER2, 611-CTF and 648-CTF promotes HER2 activity while 687-CTF is an inactive form (Jackson *et al.*, 2013; Wang *et al.*, 2013a).

Buffering systems are considered responsible for the maintenance of the network signalling when damages to individual components occur, consisting of protective mechanisms and are characteristic of robust systems. Heatshock protein-90 (HSP90) is the main chaperone that protects against damage inflicted on the HER network (Citri & Yarden, 2006). As a contrast to other EGF receptors, the HER2 is one of the most prominent kinases targeted by HSP90, through a ternary complex, the HSP90, HER2 and the co-chaperone CDC37, which stabilizes HER2 in the plasma membrane (Bertelsen & Stang, 2014). Besides the regulation of the protein stability, HSP90 also restrains HER2 kinase activity and limits its capacity to active heterodimers (Citri & Yarden, 2006).

Besides the traditional cytoplasmic HER signaling pathways, there are evidences that EGF receptors can also suffer translocation from the cell surface to the nucleus (Wang *et al.*, 2010). There are three proposed mechanisms for the transport of membrane proteins to the nucleus (Figure 10): the activity of transmembrane domain-binding chaperones, endosome-mediated nuclear translocation and retro-translocation by endoplasmic reticulum (RE)-associated trafficking machinery. The two major functions of the nuclear HER proteins are the transcriptional regulation that activate genes, such as COX-2, and the tyrosine kinase activity, due to the presence of the HER tyrosine kinase domain (Citri & Yarden, 2006; Wang & Hung, 2009; Bertelsen & Stang, 2014).

HER2 is usually regarded as a non-autonomous amplifier of the HER signalling network, rather than just an additional growth factor receptor (Tzahar *et al.*, 1996; Graus-Porta *et al.*, 1997; Citri & Yarden, 2006).

Moreover, HER2 has a basal tyrosine kinase activity relatively high and presents slow rates of growth-factor dissociation (Citri & Yarden, 2006). Thus, HER2 heterodimers are slowly internalizing and can potentiate HER signalling (mostly of EGFR), by reducing the endocytosis (Baulida *et al.*, 1996; Lenferink *et al.*, 1998). They are also more frequently recycled back to the cell surface (Citri & Yarden, 2006).

2.3. Tumorigenesis of HER2

The HER network is associated with multiple human cancers. Deregulation of the HER induced signalling pathways can promote multiple properties of neoplastic cells, including proliferation, migration, angiogenesis, stromal invasion and resistance to apoptosis (Marmor *et al.*, 2004).

The first evidence of the role of HER2 in cancer was proposed by Schechter *et al.* (1984). These investigators demonstrated an overexpression of a 185 kDa protein in carcinogen-induced neuroblastomas in rats, which was the rat ortholog *neu* of HER2. In this species, oncogenic activation of *neu* occurs as a result of a single point mutation in the transmembrane domain (described in chapter 2). This causes an increase in the tyrosine kinase activity of *neu* protein and induces ligand-independent receptor oligomerization. The overexpression of either rat or human wild-type HER2 has demonstrated ability to transform diploid cells into cancer cells that under the control of mammary-specific promoters, lead to metastatic mammary tumors in transgenic mice (Bargmann *et al.*, 1986; Bargmann & Weinberg, 1988a; Arteaga & Engelman 2014).

Slamon *et al.* (1987) were the first to report HER2 amplification in human primary breast carcinomas (in about 20 to 30% of the samples) and to associate this oncogenic alteration with poor outcome in cancer patients, suggesting a relation between HER2 amplification and cancer aggressiveness.

Therefore, amplification of HER2 gene is the main cause for HER2 overexpression. This characteristic differs from several others oncogenic proteins, where gene mutations are in the basis of tumorigenesis. Consequently, the oncogenic potential of HER2 has been extensively studied and was already demonstrated in a number of different types of cancer, as breast, lung, pancreas, oesophagus, colon, endometrium, cervix and ovarian tumors (Yarden & Sliwkowski, 2001).

HER2 oncogenic effect may be related to its high basal autophosphorylation, that results in its activation and consequently in the activation of the signalling pathways that induce transformation of the cells (Di Marco *et al.*, 1990; Lonardo *et al.*, 1990).

Alternatively, overexpressed HER2 may promote tumor formation through increased formation of homodimers or heterodimers, resulting in signal potentiation (Moasser, 2007). This signal potentiation is a consequent of the increased ligand affinity of HER2 heterodimers, and the decrease in the rate of ligand dissociation (Marmor *et al.*, 2004; Moasser, 2007).

One example of heterodimer signal potentiation is the increased heterodimerization between HER2 and EGFR that interferes with the endocytic regulation of EGFR. Firstly, EGFR-HER2 heterodimers evade more easily endocytic degradation in favour of the recycling pathway. Second, HER2 has a relatively slow rate of endocytosis which reduces the rate of EGFR internalisation and subsequent degradation (Sorkin *et al.*, 1993; Baulida *et al.*, 1996). Consequently, EGFR-HER2 heterodimers present an increased signalling duration and potency, which culminates in the increase of EGFR membrane expression and activity (Hendriks *et al.*, 2003a; Hendriks *et al.*, 2003b; Lenferink *et al.*, 1998; Waterman *et al.*, 1998).

Considering HER3, several studies indicate that HER3 is an obligate partner in HER2 induced cell transformation. HER2/HER3 heterodimer is the most transforming and mitogenic of HER receptor's combinations (Neve *et al.*, 2002). The overexpression of HER2 activates AKT through HER3 and AKT presents a central role in tumorigenesis (Holbro *et al.*, 2003; Moasser, 2007). AKT interferes in several signal transduction pathways that regulate numerous cellular functions critically important for cancer cells, including cell proliferation and survival, cell size and response to nutrient availability, glucose metabolism, epithelial-mesenchymal transition and cell invasiveness, genome stability and angiogenesis (Vivanco & Sawyers, 2002).

In addition, HER2 promotes metastatic invasion since the upregulation of HER2 promotes the secretion of basement membrane degradative enzymes, as the matrix metalloproteases (MMP) and a close relationship between HER2 and MMP-1 was already established (Ménard *et al.*, 2003; Bao *et al.*, 2010). The MMP determine modifications in the tissue architecture through the breakdown of the matrix and consequent disturbance of cell-to-cell and cell-to-matrix interactions. These alterations, together with other events, like changes in integrin and cadherin functions that are frequently observed in tumor cells with activated HER, facilitates the communication between tumor cells and the escape of microenvironment control (Ménard *et al.*, 2003).

As described for negative regulatory pathways (chapter 2.2), alternative splice variants of HER2 can also be responsible for tumorigenesis as designed by Δ16HER2, 611-CTF and 648-CTF (Wang *et al.*, 2013a). The Δ16HER2 is one of the most studied and is very similar to the full-length protein HER2, with the exception of the exon 16 that suffers a deletion in this splice-variant protein. This deletion eliminates the amino acids 634–649 in domain IV of

the protein, producing a conformational change in the ECD and the formation of constitutively activated HER2 homodimers (Jackson *et al.*, 2013; Wang *et al.*, 2013a). The Δ16HER2 presents a significantly increased tyrosine phosphorylation and increased transforming potency similar to the mutated *neu* gene in the mice (Kwong & Hung, 1998; Moasser, 2007). This naturally human HER2 RNA transcript was already documented in human cell lines of mammary carcinomas as well as in primary human breast tumors. It seems to constitute a more aggressive variant when compared to the wild-type HER2 (Scott *et al.*, 1993; Siegel *et al.*, 1999; Castiglioni *et al.*, 2006).
As for 611 and 648-CTF, they lack the ECD that allows the dimerization and subsequent activation of the HER2 protein (Anido *et al.*, 2006; García-Castillo *et al.*, 2009; Pedersen *et al.*, 2009).

Besides alternative splicing, another mechanism of CTF production is HER2 shedding (Tsé *et al.*, 2012). HER2 shedding produces truncated HER2 CTF that retains kinase activity, through proteolytic cleavage in the juxtamembrane region of HER2 (around alanine 648). This cleavage originates two products, the soluble HER2 fragment that includes the ECD portion of the protein (it can be found in serum - sHER2) and the intracellular CTF (Tsé *et al.*, 2012). The release of ECD into the extracellular medium significantly increases the tyrosine kinase activity, *in vitro*, and enhanced the transforming potency of the amino-terminally truncated HER2 ($p95^{HER2}$) that is 10 to 100 fold more oncogenic than full length HER2 (Segatto *et al.*, 1988).
In vivo, the HER2 shedding was associated with poor outcome (Carney *et al.*, 2003). The HER2 shedding results from proteolytic cleavage, and is caused by various zinc-containing metalloproteases including members of the ADAM (A Disintegrin and Metalloproteases) and MMP families (Sanderson *et al.*, 2006; Tsé *et al.*, 2012).

Finally, HER2 mutations are less common and almost exclusively observed in cancers without HER2 gene amplification. Mostly, they are missense mutations in the tyrosine kinase and extracellular domains or duplications/insertions confined to a small region within exon 20 (Buttitta *et al.*, 2006; Arteaga & Engelman, 2014, Zabransky *et al.*, 2015). These mutants increase signalling activity and are associated to lung adenocarcinoma, lobular breast, bladder, gastric and endometrial cancers, despite the oncogenic mechanisms still being unclear (Arteaga & Engelman, 2014). In fact, only a few and recent studies indicate that HER2 polymorphisms and mutations induce cellular transformation through an increase of HER2 autophosphorilation, which results in the increase of protein tyrosine kinase activity (Benusiglio *et al.*, 2005; Jo *et al.*, 2008).

2.4. HER2 and breast cancer

Breast cancer represents the most common cancer in women and the second leading cause of death among women (Raica *et al.*, 2014; Global Burden of Disease Cancer Collaboration, 2015; WHO, 2015).

HER2 oncogene is amplified in several types of cancer, including 10 to 40% of breast cancer, leading to an overexpression of the HER2 protein. The amplification is usually correlated to an increased expression of the mRNA resulting in the overexpression of the encoded protein (Wolf *et al.*, 2013). Breast cancers HER2-positives (HER2+) can have up to 25-50 copies of the HER2 gene and up to 40-100 fold increase in HER2 protein resulting in 2 million receptors expressed at the tumor cell surface (Kallioniemi *et al.*, 1992; Gutierrez & Schiff, 2011).

The importance of these protein lies in the fact that HER2 gene amplification, mRNA or protein overexpression confers a worse biological behaviour and clinical aggressiveness in breast cancer, with an increased risk of metastasis, resistance to many types of therapy and a poor prognosis (Ménard *et al.*, 2000; Gutierrez & Schiff, 2011).

Several studies unravelled data about relative risk (RR) of untreated HER2 positive breast cancer patients and adverse clinical outcome: considering the OS, the mean risk was 2.74 (range 1.39-6.93) and for DFS, the mean RR was 2.04 (range 1.3-3.01) (revised by Ross *et al.*, 2009).

Thus, gene amplification/protein overexpression is correlated with several features of tumor progression such as: tumor size, lymph node infiltration, high histologic and nuclear grade, absence of hormone receptor expression (ER and PR), DNA aneuploidy, p53 mutation, topoisomerase IIa amplification, high urokinase-type plasminogen activator (uPA) expression, high cell proliferation rate and relative resistance to endocrine therapy (Ross *et al.*, 2009; Ménard *et al.*, 2000; Gutierrez & Schiff, 2011).

As described in chapter 2.3, the aggressive behaviour of breast tumors results' from the activation of HER2 network, due to the HER2 protein overexpression. The homo or heterodimerization will triggers TK activity, resulting in the phosphorylation of tyrosine residues within the intracellular domain of either of the participating receptors. The pattern of phosphorylation defined by which tyrosines of specific HER receptors are activated, can directly influence the activation of signalling pathways. In sum, HER2 can influence multiple checkpoints that are crucial for the cell cycle (Figure 9).

Besides the homo and/or heterodimerization, the initiation of HER2 signal transduction in breast cancer is also induced via formation of the truncated HER2 CTF, which retains kinase activity and that can be generated by alternative splice variants or HER2 shedding, as described in chapter 2.3 (Tsé *et al.*, 2012).

Overall, HER2 overexpression in breast cancer induces proliferation by disrupting the function of proteins that regulate cell-cycle progression and apoptosis, especially through the PI3K and MAPK pathways that present a prolonged signal with the HER2 overexpression (Neve *et al.*, 2002). The persistent activation of the PI3K pathway promotes cell proliferation, prevents apoptosis, and increases tumor angiogenesis and transformation, while MAPK pathway upregulation induces cell proliferation and transformation (Park *et al.*, 2008). HER2 overexpression is associated with many cellular phenotypes, including increased proliferation, migration, invasion, cellular differentiation, tumor angiogenesis (via the upregulation of the VEGF) and loss of cellular adhesion. This directly leads to tumorigenesis, aggressive tumor behaviour and increased metastatic potential (Park *et al.*, 2008; Appert-Collin *et al.*, 2015).

Fortunately, this type of tumors have anticancer therapies targeted to these protein, using monoclonal antibodies that bind to the HER2 protein and inhibit the tumor cell growth (Harari & Yarden, 2000).
Therefore, it is important to accurately evaluate the status of HER2, which is recommended for primary, recurrent or metastatic tumors. Usually it is performed an immunohistochemistry (IHC), in order to detect the overexpression of the protein or an *in situ* hybridization (ISH) that allows the detection of the gene amplification (Wolf *et al.*, 2013).

2.4.1. Chromossome 17 Polysomy

Polysomy of the chromosome 17 is usually detected by ISH technique and has been reported in 10 to 50% of cases of breast cancer. This wide range of results may be a consequence of the different definitions of polysomy in these studies, varying between more than two copies of the chromosome per cell to more than four copies per cell (Torrisi *et al.*, 2007; Hyun *et al.*, 2008). Like gene amplification, polysomy of the chromosome 17 can be responsible for HER2 overexpression detected by IHC. Nevertheless, the biological and clinical relevance of these polysomy remains unclear and contradictory results were found with some studies associating chromosome 17 polysomy to hormone receptor positivity, low grade and low proliferating tumors, and other studies associating 17 polysomy to adverse histological features such as high histological grade, high nuclear grade, poor Nottingham prognostic index, advanced local tumor extent and progesterone receptor negativity (Torrisi *et al.*, 2007; Orsaria *et al.*, 2013).

2.4.2. Methods of HER2 testing

Nowadays, there are several methods to determine HER2 status, but the most used is the IHC, that evaluate HER2 protein expression. Therefore, there is a standardized interpretation method to assess HER2 expression by IHC (Wolf *et al.*, 2013). IHC has many advantages to

support its widespread adoption: the preservation of the tissue architecture, making possible the identification of local areas of overexpression within a heterogeneous sample; its applicablility to routine patient samples; being relatively inexpensive; being a simple technique and presenting an easy system to score (Rampaul *et al.*, 2002). Nevertheless, there are also several factors that can affect IHC process, especially when performed on archival, fixed, paraffin embedded tissues, as these parameters influence antigen loss (Penault-Llorca *et al.*, 1994).

Gene amplification is detected by FISH (Fluorescence *in situ* hybridization), CISH (Chromogenic *in situ* hybridization) or SISH (Silver *in situ* hybridization) (Di Palma *et al.*, 2007; Shousha *et al.*, 2009; Wolf *et al.*, 2013). These techniques are highly sensitive and employ specific HER2 probes (antisense oligonucleotides linked to a marker such as digoxigenin).

According to American Society of Clinical Oncology (ASCO) guidelines, FISH is the recommended technique for evaluation of HER2 gene amplification. It measures the number of copies of the HER2 gene, typically in conjunction with the number of chromosome 17 centromere copies, in order to differentiate gene amplification and polysomy of the chromosome 17 (Wolff *et al.*, 2013). This DNA-based assay is more stable and reproducible than IHC, however, it is a more expensive technique and labour intensive, is more difficult to interpret and requires an highly sensitive immunofluorescence microscopy. In addition, FISH assay produces fluorescent signals that will fade in a short period of time, precluding a long storage of the samples (Wolff *et al.*, 2013). All these factors led to the use of IHC as the frontline test for HER2 evaluation, and FISH as the second selected technique, performed only when IHC presents a negative or equivocal result (Wolff *et al.*, 2013).

In order to overcome these disadvantages, CISH and SISH techniques have been evaluated as potential alternatives, as they do not require an expensive fluorescence microscope with multi-band-pass filters, they produce a permanent staining and samples can be archived indefinitely. Additionally, the cell morphology is easier to analyze, particularly for distinguish invasive cancer cells and *in situ* components as well as identify tumor heterogeneity, even in low magnification, using an ordinary bright-field microscopy, which is another advantage (Rosa *et al.*, 2009; Shousha *et al.*, 2009). Using these techniques, the HER2 gene amplification is measured using a conventional peroxidase reaction (CISH) or an enzymatic metallography and metallic silver deposition (SISH) that allows the detection and enumeration of HER2 gene copy signals with simultaneous histologic examination by ordinary bright-field microscopy (Di Palma *et al.*, 2007; Carbone *et al.*, 2008; Shousha *et al.*, 2009).

Both HER2 overexpression and HER2 gene amplification are associated with poor prognosis, having prognostic and predictive value, once they identify the women that

potentially benefits from HER2-targeted therapies (Slamon *et al.*, 1987; Press *et al.*, 1997; Leary *et al.*, 2009).

These techniques are useful for selecting patients with primary breast cancer that are eligible for targeted HER2-therapy. Nevertheless, they are not adequate for assessing the HER2 status of patients after the tumor is removed as discordant results between the HER2 expression of the primary and the recurrent/metastatic lesions have been reported (Leary *et al.*, 2009; Niikura *et al.*, 2012; Turner & Di Leo, 2013). In addition, the currently available tissue diagnostic methods for HER2 have their own limitations, such as inter-laboratory variability (Roche *et al.*, 2002), discordance between diagnosis by IHC and FISH (Dybdal *et al.*, 2005), or antigen loss associated with paraffin archived tissue (Wolff *et al.*, 2013).

Such considerations have promoted an increasing interest in quantifying the serum extracellular domain fragment of HER2 (HER2-ECD). Enzyme-Linked Immunosorbent Assay (ELISA) is used for quantification of the soluble circulating HER2 ECD in serum or plasma. As described in chapter 2.3, sHER2 results from the cleavage of the ECD of HER2 that is released into the extracellular medium thus entering into blood circulation, being present at low levels in the serum of healthy individuals. Conversely, the sHER2 levels are elevated in various cancers, mainly in breast adenocarcinomas (Carney *et al.*, 2003).

ELISA may enable the monitoring of the disease progression and patient’s response in real time, adding relevant additional information. Additionally, it could complement tissue testing, improving the evaluation of the HER2 status. In the metastatic relapse sHER2 presents obvious advantages over IHC or FISH, because the biopsy of the metastatic lesion is no longer necessary to evaluate protein status. In summary, sHER2 quantification allows the assessment of HER2 status variations in real time (Tsé *et al.*, 2012).

ELISA was approved by USA Food and Drug Administration (FDA) in 2000 and the current approved cut-off value for increased sHER2 concentration is 15 ng/ml (Moelans *et al.*, 2011). Moderate sHER2 increase (not exceeding 50 ng/ml) was also described in the absence of cancer disease, mostly in association with liver disease, preeclampsia and chronic heart failure (Tsé *et al.*, 2012).

Researchers concluded that tissue HER2 status and serum HER2 levels presented a good correlation in early and metastatic breast cancer (MBC) (Ludovini *et al.*, 2008; Witzel *et al.*, 2010; Tsé *et al.*, 2012). In addition they also associated sHER2 with a worse prognosis (review in Carney *et al.*, 2003; Moelans *et al.*, 2011; Tsé *et al.*, 2012) and demonstrated that sHER2 has predictive value (Köstler *et al.*, 2004; Mazouni *et al.*, 2007; Witzel *et al.*, 2010). However, the ASCO did not recommend the quantification of sHER2 in the routine management of individual patients with breast cancer (Leary *et al.*, 2009).

The quantitative real time reverse transcript polymerase chain reaction (qRT-PCR) is a quantitative method that successfully identifies the high expression of HER2 mRNA. This method has the advantage of being insensitive to inter-observer variability and

standardization can be possible (Susini *et al.*, 2010). However, qRT-PCR presents important drawbacks that prevent its routine use in diagnostic laboratories, such as the need of isolation of the tumor cell population within the tissue and the template quality (Rosa *et al.*, 2009). Nevertheless, studies show a good correlation between qRT-PCR and both IHC and ISH techniques even in paraffin-embedded material (Bergqvist *et al.*, 2007; Barberis *et al.*, 2008; Rosa *et al.*, 2009; Susini *et al.*, 2010). Additionally, qRT-PCR is increasingly used in the form of a nested PCR for measuring mRNA levels in circulating tumor cells (CTCs). However, the controversial results prevents its recommendation by current guidelines (Harris *et al.*, 2007; Castle *et al.*, 2014).

The HER2 mRNA expression is also evaluated by other assays that analyze the expression of a panel of genes, such as Oncotype DX assay (Genomic Health, Redwood City, CA, USA), TargetPrint, Mammaprint and the BluePrint microarrays (Agendia, Irvine, CA, USA) and Prosigna (NanoString Tecnologies, Seatle, WA, USA).

Oncotype DX assay (Genomic Health) uses qRT-PCR and quantifies the expression of 21 genes in women with newly diagnosed early stage breast cancer (Paik *et al.*, 2004; Moelans *et al.*, 2011; Zanotti *et al.*, 2014; Grant *et al.*, 2015). TargetPrint (Agendia) is a microarray that measures mRNA levels of HER2, ER and PR (Roepman *et al.*, 2009; Nguyen *et al.*, 2012). Mammaprint and BluePrint microarrays (Agendia) are RNA microarray that evaluate 70-gene and 80-gene profiles, respectively (Glas *et al.*, 2006; Krijgsman *et al.*, 2012; Nguyen *et al.*, 2012; Zanotti *et al.*, 2014). Finally, Prosigma (NanoString) is a genomic test based on the 50-gene subtype predictor and uses hybridization reaction to measure the RNA expression of the 50 genes, providing a “Risk of recurrence” score (Zanotti *et al.*, 2014).

Several other methodologies can be used to determine the HER2 status in women with breast cancer, although they are not routinely used in clinical management of patients with breast cancer (review in Ross *et al.*, 2009; Moelans *et al.*, 2011). For HER2 gene amplification detection southern blot, slot blot analysis, qPCR and multiplex ligation-dependent probe amplification (MLPA) can also be used (Slamon *et al.*, 1987; Tsuda *et al.*, 1989; Borg *et al.*, 1990; Clarkn& McGuire, 1991; Schouten *et al.*, 2002; Tse *et al.*, 2005; Moelans *et al.*, 2009). To determine mRNA overexpression Northern blot analysis, slot blot analysis and ISH can be performed (Slamon *et al.*, 1989; Ross *et al.*, 2009).

Finally, western blot can assess the HER2 protein levels (Slamon *et al.*, 1989; Borg *et al.*, 1990). Recently, a new protein quantification method was developed by precisely quantifying the total HER2 expression and HER2 homodimers (HERmark® assay, Monogram Biosciences, South San Francisco, CA, USA). The HERmark® assay enables accurate quantification of proteins and protein-protein complexes through proximity-based immunoassay approach, using a dual antibody with VeraTag (Monogram Biosciences) technology (Shi *et al.*, 2009; Huang *et al.*, 2010; Larson *et al.*, 2010; Yardley *et al.*, 2015).

Another technique that allows the evaluation of the protein functional status is the phosphorylated HER2 receptor assay, that uses IHC to determine autophosphorylated HER2 (DiGiovanna & Stern, 1995; DiGiovanna *et al.*, 1996; Ross *et al.*, 2009).

2.4.3. HER2 target therapies

The development of HER2 target therapies has revolutionized the treatment of women with HER2-positive breast cancer and has significantly improved their outcomes.

Over the last years, a substantial number of agents that target HER2 have been developed and the main drugs are summarize in Table 4 (Ahmed *et al.*, 2015). The two main strategies used to target HER2 (and the others members of EGFR family), are monoclonal antibodies (mAbs), that bind to the receptor's extracellular region and small-molecule tyrosine kinase inhibitors (TKIs), that block signal transduction, as represented in Figure 11 (Monteiro *et al.*, 2015).

Table 4. HER2 targeted therapies approved in HER2 positive breast cancer (Monteiro *et al.*, 2015)

Class	Agent	
Monoclonal antibodies		
	Trastuzumab	A humanized monoclonal antibody that binds to the extracellular domain IV of HER2 and prevents ligand-independent HER2 signalling.
	Pertuzumab	A humanized monoclonal antibody that binds to the extracellular domain II of HER2 and inhibits ligand dependent HER2-HER3 dimerization.
Antibody drug conjugates		
	Ado-trastuzumab emtansine	An antibody-drug conjugate consisting of the cytotoxic agent DM1 linked to trastuzumab.
Small molecules inhibitors		
	Lapatinib	An oral dual EGFR/HER2 reversible tyrosine kinase inhibitor both EGFR and HER2 that supresses the downstream pathways.
	Afatinib Neratinib	Irreversible tyrosine kinase inhibitor of EGFR/HER2/HER4.

DM1 - Maytansine

2.4.3.1. Monoclonal antibodies

Several murine monoclonal antibodies against the extracellular domain of the HER2 protein have been found to inhibit proliferation of cells overexpressing HER2 (Shepard *et al.*, 1991). Trastuzumab (Herceptin, Genentech, South San Franscisco, USA) was the first mAb approved by the FDA for breast cancer treatment, in 1998 (Perez & Baweja, 2008). It resulted from the fusion of the antigen-binding region with the framework region of the human IgG, in order to minimize the immunogenicity. Trastuzumab has a high affinity for domain IV of the extracellular domain of the HER2 receptor (Figure 11) preventing the activation of its intracellular tyrosine kinase (Hudis, 2007). There are several mechanisms of action of this drug: prevention of the homodimerization and the heterodimerization (especially

the HER2-HER3 dimerization), induction of endocytic destruction of the receptor, induction of antibody-dependent cellular cytotoxicity (ADCC) and inhibition of extracellular domain cleavage that consequently inhibits the formation of p95 (Molina *et al.*, 2001; Arnould *et al.*, 2006; Hudis, 2007; Jones & Buzdar, 2009; Monteiro *et al.*, 2015).

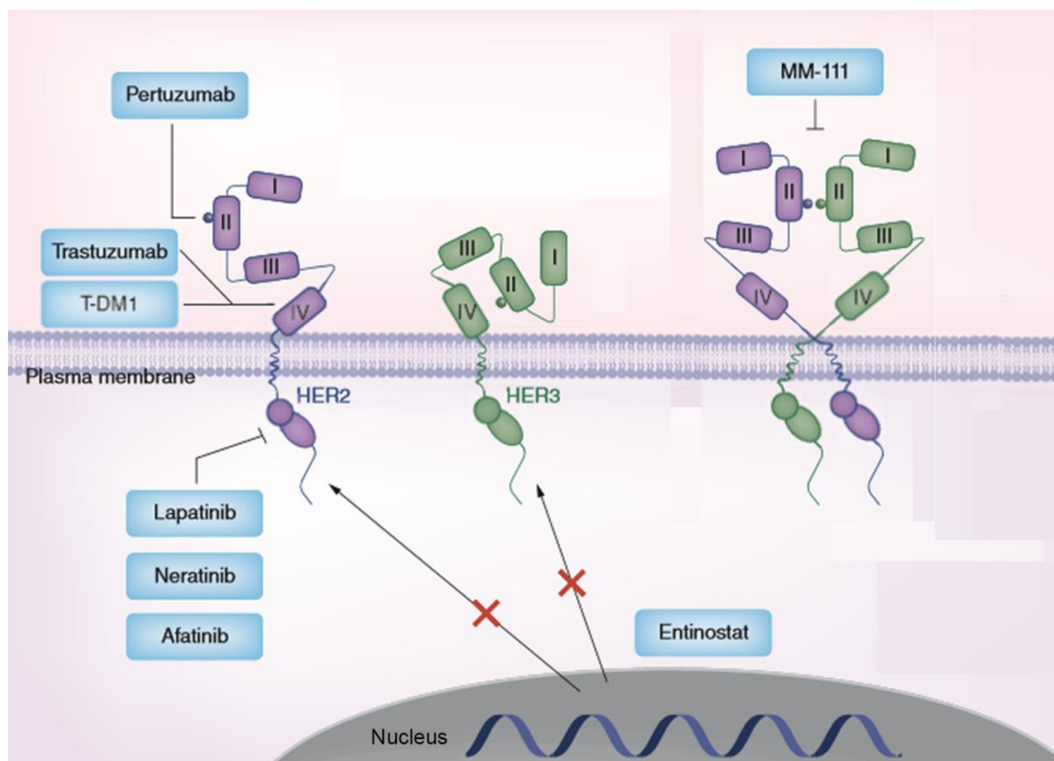

Figure 11. Extracellular and intracellular HER2 target therapies.

There are two main groups of drugs represented: the monoclonal antibodies, directed against ECD and the tyrosine kinase inhibitors targeting the ICD. The monoclonal antibodies group is represented by the trastuzumab, T-DM1 (Ado-trastuzumab emtansine) and pertuzumab. They exclusively target the HER2 protein. The MM-111 targets HER2 and HER3 proteins. Alternatively, the TKIs group is represented by lapatinib that also possesses action against EGFR, besides HER2 and by neratinib and afatinib that target the TK of HER1/HER2 and HER4. Finally, entinostat is also illustrated in the image. Entinostat is a histone deacetylase inhibitor (HDACi) that dually targets HER2 and HER3. *Adapted from* Monteiro *et al.* (2015)

Trastuzumab has demonstrated efficacy in HER2-positive advanced breast cancer and was initially approved for metastatic breast cancer, in combination with chemotherapy or as monotherapy (Slamon *et al.*, 2001; Monteiro *et al.*, 2015). It was subsequently evaluated in early stage breast cancer and nowadays is predominantly used in combination with chemotherapy due to its clear advantage in improving clinical outcome (Perez & Baweja, 2008; Monteiro *et al.*, 2015).

Considering the side effects, initially trastuzumab was associated with a variety of adverse events, including pain, gastrointestinal disturbances, minor haematological deficiencies,

pulmonary symptoms and congestive heart failure (Stebbing *et al.*, 2000; Hudis, 2007; Ross *et al.*, 2009). From these symptoms, cardiac toxicity remains the most significant limiting factor for trastuzumab use, especially if patients have been subjected to prior or concomitant treatment with anthracycline drugs that are also associated with heart damage (Ross *et al.*, 2009; Sandoo *et al.*, 2015). Nonetheless, trastuzumab related cardiac dysfunctions are typically reversible with the standard medical management (Hudis, 2007; Monteiro *et al.*, 2015). Therefore, cardiac monitoring is recommended for patients receiving treatments with this mAb (Seidman *et al.*, 2002).

Despite the tremendous improvement in overall survival and disease free survival times of the HER2 positive breast cancer patients treated with trastuzumab, not all HER2-positive patients respond to treatment and the development of resistance is an issue (Rosen *et al.*, 2010). Potential mechanisms of resistance to trastuzumab include bypass mechanisms, mutations of the HER2 target, masking of HER2 proteins, modulation of the activity of intracellular signalling proteins and PTEN deficiency (Nahta *et al.*, 2006; Jones & Budzar, 2009; Monteiro *et al.*, 2015).

In order to overcome this resistance, several other molecules have been developed, including pertuzumab (Figure 11). Pertuzumab (rhuMab 2C4, Omnitag™; Genentech) is a recombinant, humanised mAb that targets the extracellular domain II of HER2 (Ahmed *et al.*, 2015). It is considered a HER dimerisation inhibitor as it blocks the ability of HER2 to heterodimerise with other members of the EGFR family thus preventing signal transduction (Jones & Budzar, 2009). Like trastuzumab, pertuzumab causes an ADCC reaction, although it does not block HER2 shedding (Ross *et al.*, 2009; Monteiro *et al.*, 2015). Once it targets a different epitope, it can be used in combination with trastuzumab, and preclinical data showed synergy between trastuzumab and pertuzumab, overcoming the mechanisms of resistance in trastuzumab-resistant patients (Jones & Budzar, 2009; Ahmed *et al.*, 2015; Monteiro *et al.*, 2015). Additionally, phase II and III clinical trials have demonstrated a significant improvement in prognostic outcome in patients with advanced breast cancer, when the association of pertuzumab and trastuzumab was used as first line treatment (Walshe *et al.*, 2006; Jones & Budzar, 2009). These results supported the approval of pertuzumab use in combination with trastuzumab in breast cancer patients, by the USA FDA (Dawood & Sirohi, 2015). Regarding the sides effects, diarrhoea, fatigue, nausea, vomiting and rash are between the most common (Jones & Budzar, 2009).

Antibody-drug conjugates (ADCs) are developed to deliver a highly potent cytotoxic agent to specific target cells and thus potentially improving the therapeutic dose of the agent (Krop *et al.*, 2010). The key components of an ADC are the cytotoxic agent, a monoclonal antibody targeting a tumor-enriched or a tumor-specific antigen and a linker that covalently binds these components together.

For HER2-positive tumors, it was developed Ado-trastuzumab emtasine (T-DM1). It consists in a trastuzumab linked to a derivate of maytansine (DM1), a potent microtubule polymerization inhibitor (Krop *et al.*, 2010; Burris *et al.*, 2011; Girirsh *et al.*, 2012). Currently, several clinical trials evaluate the efficacy of adjuvant T-DM1 in women with HER2 positive breast cancer (reviewed in Oostra & Macrae, 2014; Ahmed *et al.*, 2015; Monteiro *et al.*, 2015). T-DM1 (Kadcyla, Genetech) includes all the mechanisms of action of trastuzumab and is still active against heavily pretreated HER2 positive MBC, including cancers that still progressed with trastuzumab combined treatment. T-DM1 significantly improved the DFS and OS, with less toxicity, when compared with lapatinib and capecitabine (Verma *et al.*, 2012). Based on these studies, FDA approved T-DM1 for patients with HER2-positive MBC which previously received trastuzumab and taxane therapy (Oostra & Macrae, 2014).

Resistance to trastuzumab could also be overcome by formulating drugs with a dual mechanism of action, such as ertumaxomab or MM-111 (Jones & Budzar, 2009; Monteiro *et al.*, 2015). Ertumaxomab is a monoclonal trifunctional and bispecific antibody that binds both HER2 and CD3. This allows the formation of a complex of T cells, HER2 positive tumor cells and macrophages or dendritic cells, leading to the phagocytosis of the tumor cells. *In vitro* studies demonstrated that ertumaxomab is effective in killing HER2 positive cells, and phase II clinical trials are currently underway (Jones & Budzar, 2009). MM-111 is a bispecific antibody designed to target HER2 and HER3 (Figure 11). MM-111 forms a trimeric complex (MM-111, HER2 and HER3) that blocks the ligand-induced activation of heterodimers. In preclinical studies MM-111 was more effective at inhibiting tumor cells growth when compared to trastuzumab plus pertuzumab and proved to increase PIK3-AKT pathway suppression when used in combination with lapatinib. Consequently, this antibody is currently under study in combination with several anti-HER2 regimens and phase I trial has already revealed that MM-111 is well tolerated (Monteiro *et al.*, 2015).

2.4.3.2. Small molecules inhibitors

Small-molecule TKIs competes with ATP at the cytoplasmic catalytic kinase domain, inhibiting tyrosine phosphorylation and blocking signal transduction. The kinase domain of the four members of EGFR family show a high degree of homology (59-81%) and the major differences are found in C-terminal residues (11-25% identity), which justify the multiple targets of these drugs (Schroeder *et al.*, 2014).

Lapatinib (Tykerb, GlaxoSmithKline, Brentford, UK) is an oral reversible TKI that competes with ATP for the ATP-binding site in HER2 and HER1 tyrosine kinases (Monteiro *et al.*, 2015). This small-molecule is a dual inhibitor of the EGFR and HER2 tyrosine kinases, which supresses the downstream signalling involving MAPK/Erk1/2 and PI3K/Akt pathways (Medina & Goodin, 2008; Ahmed *et al.*, 2015). Once it uses a different pathway to interfering with HER2 network, Lapatinib is still effective against trastuzumab-resistant breast cancer

cells (Konecny *et al.*, 2006), and the combined use of (lapatinib and trastuzumab) enhances trastuzumab efficacy (Ahmed *et al.*, 2015).

Besides the combination of lapatinib with trastuzumab, researchers have also demonstrated the efficacy and safety of lapatinib either as monotherapy or in combination with chemotherapy or endocrine therapy in patients with MBC (Cetin *et al.*, 2013; Monteiro *et al.*, 2015). An example is the combination of lapatinib and capecitabine that has proven to significantly improve DFS and OS without an increase of the secondary effects, when compared with the use of capecitabine alone. Lapatinib and capecitabine were approved by FDA for the treatment of advanced breast cancer or MBC in patients that do not respond to trastuzumab (Geyer *et al.*, 2006; Cetin *et al.*, 2013; Monteiro *et al.*, 2015).

Finally, lapatinib is a good candidate to targeting HER2-positive central nervous system (CNS) metastases, as it presents a low molecular weight, and therefore being easier to cross the blood-brain barrier wall (Lin *et al.*, 2009; Bachelot *et al.*, 2013). Recently, Lin *et al.* (2009) confirmed that lapatinib present a modest antitumor activity in CNS.

Others TKIs include Afatinib and Neratinib that are irreversible binders of the HER receptors (HER1, HER2 and HER4) and have demonstrated efficacy in HER2 positive breast cancers, with especial interest in patients resistant to lapatinib and/or trastuzumab therapy (Ahmed *et al.*, 2015; Hirsh 2015; Monteiro *et al.*, 2015; Segovia-Mendonza *et al.*, 2015).

2.4.3.3. Other HER2 target therapies

Several other target therapies are under development or being tested, either directly against HER2 either targeting other proteins that interfere with the HER2 signaling network.

Entinostat (SNDX-275) is a Histone deacetylase inhibitor (HDACi) that targets both HER2 and HER3 (Figure 11). Although the exact mechanism of action is still unclear, entinostat seems to induce the transcription of microRNAs (mRis) that silences the expression of HER2 and HER3 (Wang *et al.*, 2013b). Moreover, researchers have demonstrated that entinostat to enhanced trastuzumab efficacy against HER2 positive breast cancer cells and Phase I trials are currently undergoing (Kummar *et al.*, 2007; Huang *et al.*, 2011).

HER2 vaccines are a novel approach toward the treatment of HER2-positive breast cancer (Ross *et al.*, 2009; Ahmed *et al.*, 2015).The existence of HER2-positive breast cancer patients that developed spontaneous anti-HER2 specific immunity with both cellular and humoral responses, launched HER2 as a possible suitable target for active immunotherapy (Ahmed *et al.*, 2015). Early data from trials studying the potential use of HER2-based vaccines in high-risk patients have shown promising results (Peoples *et al.*, 2008).

Several studies are currently undergoing to develop and evaluate new therapeutic options in HER2-positive breast cancer, targeting several crucial proteins in the HER network, such as

HSP90 inhibitors (e.g.: geldanamycin and tanespimycin), PI3K inhibitors (e.g.: everolimus, an mTOR inhibitor), JAK 1 and JAK 2 inhibitors or inhibitor of the insulin-like growth factor 1 (IGF1) receptor, among others (Modi *et al.*, 2007; Jones & Budzar, 2009; Ross *et al.*, 2009; Ahmed *et al.*, 2015; Monteiro *et al.*, 2015). Regarding HSP90 inhibitors, they have presented a good safety and tolerability profile in a first report of a trial of tanespimycin combined with trastuzumab in advanced pretreated MBC (Modi *et al.*, 2007).

2.4.4. HER2 metastatic disease

Before the use of HER2 targeted therapy, the HER2 positive primary tumors were more likely to early metastize to major visceral sites, including axillary lymph nodes, bone marrow, lungs, liver, adrenal glands and ovaries (Ross *et al.*, 2009). After the use of HER2 targeted therapy, the progression to visceral sites has diminished and the development of clinically significant CNS metastatic disease has been observed (Bendell *et al.*, 2003; Pestalozzi *et al.*, 2006, Lin & Winer, 2007). Nevertheless, Pestalozzi and colleagues (2006) identified HER2 as a risk factor for the development of metastasis in CNS, independently of the treatment.

The two main reasons for brain metastases in HER2 positive MBC are the improvement of visceral disease with the use of trastuzumab (leading to an increase of the OS time in treated patients), and the blood-brain barrier wall that prevents trastuzumab to penetrate in CNS (Lin & Winer, 2007).

2.5. Feline homologue of HER2 (*f*HER2)

In cats, the *f*HER2 oncogene is located in chromosome E1 and comprises 28 exons (GenBank: NC_018736.2). The *f*HER2 transcript presents 3783 bp (GenBank: AY702651) and the corresponding translated protein possesses 1260 aa (GenBank: AAW23986.1). In cats, it was established that *f*HER2 protein presents an extracellular, a transmembrane and an intracellular domain with tyrosine kinase activity, like described for humans (de Maria *et al.*, 2005). De Maria *et al.* (2005) demonstrated a great similarity (92%) between human 17 and 23 exons and the corresponding feline sequence. These authors found an 88% and a 94% similarity with the corresponding protein of mouse and dog, respectively.

Santos *et al.* (2012) have studied the *f*HER2 fragments encompassing exon 17 to 20 that encode the following regions of the *f*HER2 protein: outer justamembrane (exon 17), transmembrane (exon 17), inner justamembrane (exon 17 and 18) and a part of the ICD (exons 18, 19 and 20). When the feline encoded sequences were compared to the correspondent human HER2 sequences, they demonstrated a higher percentage of similarity. In addition, the same group of researchers (Santos *et al.*, 2013) have analyzed the DNA fragment from exons 10 to 15 that codes a portion of the ECD of HER2 protein and also discover a high similarity with the correspondent human sequence. When analyzed the

protein sequence correspondent to exon 10 to 15, a similarity of 92% was obtained between the human and cat HER2 protein.

2.5.1. Feline mammary tumors

In the case of *f*HER2 in FMC, the existing studies show inconsistent results with a wide range of *f*HER2 positivity (5.5% to 90%) and no association with gene amplification, contrasting to what is described for humans (De Maria *et al.*, 2005; Millanta *et al.*, 2005a; Winston *et al.*, 2005; Ordás *et al.*, 2007; Rasotto *et al.*, 2011).

Furthermore, De Maria *et al.* (2005) demonstrated a good correlation between mRNA expression and protein expression in FMC.

Santos *et al.* (2013) detected amino acid variants in the extracellular region of the *f*HER2 protein, in the region where it would probably occur the interaction of *f*HER2 with the therapeutic antibody trastuzumab. Additionally, the gene expression analysis suggests the presence of *f*HER2 gene post-transcriptional regulation and the occurrence of proteins with truncations and single point mutations in FMC.

The clinicopathological role of *f*HER2 in FMC is still unclear. Millanta *et al.* (2005a) correlated the overexpression of *f*HER2 in FMC with a lower OS. In this study, cats with negative *f*HER2 status present a mean survival time of 18.7 months while cats with *f*HER2 positive MT achieved an OS mean of 14.6 months. In contrast with these results, Santos *et al.* (2013) detected a positive correlation between a higher *f*HER2 mRNA expression and a better clinical outcome.

3. Molecular classification

Classically, several features are used to predict the outcome and treatment response in breast cancer such as age, tumor size, axillary node involvement, angiolymphatic invasion, histological grade, hormonal receptor status and HER2 amplification (Bosch *et al.*, 2010). However and considering the heterogeneity of human breast cancer, this classification was not sufficient as a prognostic tool. Consequently, cDNA microarrays techniques and hierarchical clustering have recently been used in studies to determine the gene expression of breast cancer. The results of these techniques allow the definition of different “molecular portraits” that present differences in the clinical outcome for each molecular portrait (Perou *et al.*, 2000).

In fact, microarray-based gene expression profiling is based on the analysis of thousands of genes in a single experiment. The relative expression of each gene represented by a probe in the microarray is analyzed through biostatistical and bioinformatic methods. After this, algorithms are applied in order to class the tumor into a cluster or a subtype (Michiels *et al.*, 2007; Weigelt *et al.*, 2010).

Using these approaches, the initial studies divided mammary tumors in four groups: luminal-like, HER2-positive, basal-like and normal-like subtypes (Perou *et al.*, 2000), and afterwards into five tumors subtypes: luminal A, luminal B, HER2-positive, basal-like and normal-like subtypes (Sørlie *et al.*, 2001). These subtypes proved to be different in terms of biology, survival and recurrence rate (reviewed in Sotiriou & Pusztai, 2009; Reis-Filho *et al.*, 2010; Vollan & Caldas, 2011). More recently, Prat and colleagues (2010) had defined a sixth subtype named claudin-low, which is described below (chapter 3.2.4).

For the identification of the different molecular profiles, numerous multigene signatures have emerged, each based on the expression levels of different sets of genes, such as MammaPrint (an assay with a 70-gene prognostic signature), Oncotype DX (21-gene signature), Genomic Grade Index (GGI, 97-gene signature), among others (van de Vijver *et al.*, 2002; Hu *et al.*, 2006; Colombo *et al.*, 2011; Metzger-Filho *et al.*, 2013).

Despite the irrefutable importance of cDNA microarrays, their high cost and complexity make their use in routine clinical practice more difficult (Jacquemier *et al.*, 2005). Alternatively, IHC is relatively inexpensive, easy to perform and well established in standard clinical pathology laboratories providing a potential surrogate to cDNA microarrays. Consequently, researchers concluded that gene expression subtypes could be identified by protein expression of the respective genes (Nielsen *et al.*, 2004; Abd El-Remim *et al.*, 2005; Jacquemier *et al.*, 2005; Goldhirsch *et al.*, 2013).

Besides the prognostic value, the definition of the molecular subtypes has implications in the therapeutic approach of breast cancer. Actually, St Gallen International Expert Consensus panel for early breast cancer recognized the usefulness of this classification in the therapeutic decision process and management of the breast cancer patient, either using genetic array testing or immunohistochemistry for defining the molecular subtypes. The Panel established five clinic-pathological subgroups, luminal A, luminal B/HER2-negative, luminal B/HER2-positive, HER2 positive-non luminal and triple negative (TN). The most important molecular subtypes are described in detail below (Goldhirsch *et al.*, 2013).

3.1. Luminal subgroup

Luminal subgroup comprises Luminal A and Luminal B subtypes. The tumors of this subgroup show mRNA and protein overexpression of the HRs (ER and/or PR) and also of the cytokeratins (CK) 8/18 that are typical associated with the luminal epithelial cells (Sørlie *et al.*, 2001; Gusterson *et al.*, 2005; Creighton, 2012).

3.1.1. Luminal A

The luminal A breast cancer is the most common subtype, representing 50-60% of the breast cancers. It is characterized by the overexpression of ER gene and ER-related genes such as GATA3, FOXA1 and XBP1, by the expression of genes usually expressed in the luminal epithelium of the mammary ducts such as CK 8 and 18 and by a low expression of genes related to cell proliferation (Perou *et al.*, 2000; Hu *et al.*, 2006; Lam *et al.*, 2014). When compared to other subtypes, Luminal A is the subtype with the lowest p53 rate of mutations (Lam *et al.*, 2014).

Based on the protein profile, St Gallen International Expert Consensus guidelines established that luminal A subtype is characterized by the overexpression of ER and/or PR, the absence of HER2 and low proliferation rates (usually evaluated through Ki-67 index). The cut point between high and low Ki-67 index levels varies between studies but 14% is the more consensual cut-off (Goldhirsch *et al.*, 2013).

Furthermore, Bcl-2, FOXA1 and CK 8/18 are also upregulated in luminal A tumors and can be used as additional biomarkers (Eroles *et al.*, 2012; Lam *et al.*, 2014).

Clinically, luminal A subtype are associated with low histological grade tumors and some studies related to several histological types like lobular carcinoma, tubular, micropapillary, mucinous and neuroendocrine carcinomas (Lam *et al.*, 2014; Vuong *et al.*, 2014). Patients with this subtype of cancer have a good prognosis, with lower relapse rates and higher survival times when compared to the other groups (Perou *et al.*, 2000; Kennecke *et al.*, 2010; Eroles *et al.*, 2012; Voung *et al.*, 2014).

Regarding the metastatic disease, there is a distinctive pattern of recurrence for luminal A primary tumors, with higher incidence of bone metastases and a lower risk of developing CNS, liver and lung metastases (Kennecke *et al.*, 2010).

Besides the prognostic value, molecular subtypes also present an important predictive value with specific therapies associated to each molecular profile. Therefore, luminal A tumors specifically benefit from endocrine therapy. According to St Gallen International Expert Consensus guidelines endocrine therapy is recommended for these patients and is often used alone, once these patients are less responsive to adjuvant chemotherapy (Berry *et al.*, 2006; Goldhirsch *et al.*, 2013; Inic *et al.*, 2014). There are three major classes of endocrine therapy drugs: the hormonal aromatase inhibitors (AI) such as anastrozole, letrozole or exemestane; the selective estrogen receptor modulators (SERMs) such as tamoxifen; and the pure selective regulators of ER like fulvestrant (Guarneri & Conte, 2009; Curigliano, 2012; Eroles *et al.*, 2012).

3.1.2. Luminal B

Luminal B tumor comprises 10 to 20% of all breast cancers and presents substantial differences to Luminal A subtype, showing a considerable different gene (and protein) expression profile and a substantially worse outcome (Sørlie *et al.*, 2001; Cheang *et al.*, 2009; Creighton, 2012).

Usually, luminal B presents the most aggressive phenotype of the Luminal subgroup, with higher histological grade and a higher proliferative index. In addition, patients with luminal B tumors are associated with a lower DFS when compared to luminal A tumors (Kennecke *et al.*, 2010; Eroles *et al.*, 2012).

Biologically, luminal B shares some gene patterns with luminal A, as the overexpression of ER gene, FOXA1 and Bcl-2 and also shares some patterns with basal-like including overexpression of proliferation markers as Ki-67 gene, survivin gene (BIRC5) and cyclin B1 gene (Creighton, 2012). Aneuploidy and loss of PR expression are also described (Creighton, 2012; Goldhirsch *et al.*, 2013). Additionally, mutation in p53 and PIK3CA genes, as well as modifications in MAPK pathway, are common (Creighton, 2012; Lam *et al.*, 2014; Vuong *et al.*, 2014).

EGFR and HER2 can also be overexpressed. Actually, approximately 30% of the tumors in the luminal B cluster present overexpression of HER2 and its associated genes, which originate the division into luminal B/HER2-negative and luminal B/HER2-positive subtype (Eroles *et al.*, 2012; Lam *et al.*, 2014).

Thus, at protein level, luminal B tumors overexpress ER and PR proteins such as luminal A subtype but present differences in Ki-67 index, for the luminal B/HER2-negative subtype, and in HER2, for the luminal B/HER2-positive subtype (Eroles *et al.*, 2012). According to St Gallen International Expert Consensus guidelines, luminal B/HER2-negative is defined as ER positive, HER2 negative and at least one of the following features: negative or low expression of PR and/or high levels of Ki-67 index. The luminal B/HER2-positive tumor presents overexpression of ER and HER2 (ER+/HER2+), independently of the PR and Ki-67 expressions (Goldhirsch *et al.*, 2013).

As it was described for luminal A, the most common location for metastatic disease in patients with primary luminal B tumors is the bone tissue. Additionally, the patients with luminal B primary tumors also present a higher recurrence of liver metastases, especially when compared to luminal A subtype (Kennecke *et al.*, 2010).

Unlike luminal A type tumors, luminal B subtype is less endocrine sensitive (Goldhirsch *et al.*, 2013). Alternatively, they present a better response to neoadjuvant chemotherapy achieving a complete response in about 17% of the luminal B tumors, which is clearly lower than for the HER2-positive and basal-like tumors (Eroles *et al.*, 2012; von Minckwitz *et al.*, 2012). Considering the subdivision of the luminal B subtype, the St Gallen panel recommends endocrine therapy with or without chemotherapy (generally containing anthracyclines) for

luminal B/HER2-negative tumors. For luminal B/HER2-positive carcinomas, the panel recommends the use of endocrine therapy plus chemotherapy plus anti-HER2 therapies (Goldhirsch *et al.*, 2013).

In order to develop more effective therapies, molecular profiling is being performed to identify novel therapeutic candidates. Interestingly, the latest studies suggest that luminal B subtypes could switch from using the estrogen signaling pathway to the use of alternative estrogen-independent pathway like the EGF signalling pathways (HER2, EGFR, IGF and AKT/PI3K/mTOR), allowing the study of new and different therapeutic targets (Prat & Perou, 2011; Creighton, 2012).

3.2. Non-luminal subgroup

The non-luminal subgroup includes the HER2-positive non-luminal subtype and the triple negative subtype. The triple negative can also be divided into the basal-like subtype, the normal-like and, more recently, the claudin-low subtype (Eroles *et al.*, 2012).

3.2.1. HER2-positive – non luminal

HER2-positive non luminal, or HER2-positive, or HER2 enriched subtype corresponds to 15 to 20% of the mammary carcinomas in women. This subtype is characterized by a high expression of the HER2 gene and other genes associated with the HER2 pathway and/or to HER2 amplicon such as topoisomerase IIa or GRB7 (Weigelt *et al.*, 2010; Eroles *et al.*, 2012; Ishikawa *et al.*, 2014; Lam *et al.*, 2014). Alternatively, they present a high rate of p53 (40-70%) and PIK3CA (39%) mutations (Eroles *et al.*, 2012; The Cancer Genome Atlas Network, 2012).

HER2-positive subtype present a low expression of the HRs related genes, an overexpression of cellular proliferation markers genes and a low expression of the characteristic luminal genes (Hu *et al.*, 2006; Huber *et al.*, 2009; Eroles *et al.*, 2012).

Using IHC, most of these tumors present a negative ER and PR status and an overexpression of the HER2 protein (ER-/PR-/HER2+), which defines the HER2-positive subtype according to the latest St Gallen International Expert Consensus guidelines (Goldhirsch *et al.*, 2013).

Clinically, the HER2 subtype is a high proliferative and high grade tumor, being associated with poor prognosis, with shorter DFS and OS, especially when compared to the luminal subgroup (Huber *et al.*, 2006; Eroles *et al.*, 2012).

As described in chapter 2.4.3, target anti-HER2 therapies have substantially improved survival in metastatic and early disease (Slamon *et al.*, 2001; Gianni *et al.*, 2011).

In summary, St Gallen International Expert Consensus guidelines recommend the use of anti-HER2 therapies with adjuvant chemotherapy, once HER2 subtype presents a high

chemosensitivity, especially for anthracyclines regimens, when compared to luminal tumors (Gennari *et al.*, 2008; Higgins & Baselga, 2011; Goldhirsch *et al.*, 2013).

3.2.2. Triple negative

3.2.2.1. Basal-like

Similarly to the other subtypes, basal-like represents 10 to 20% of all breast carcinomas (Eroles *et al.*, 2012). This subtype has a great diversity of presentations, regarding the histopathological feautures, mutation profiles, response to chemotherapy, metastatic behaviour and survival rates (Vuong *et al.*, 2014).

These tumors are characterized by expressing genes usually present in normal breast myoepithelial cells, including high molecular weight cytokeratins (as CK 5/6 and CK 14), p-cadherin, caveolin 1 and 2, nestin, CD44 and EGFR (Nielsen *et al.*, 2004; Eroles *et al.*, 2012; Vuong *et al.*, 2014). Basal tumors can also express genes characteristic of luminal epithelium like CK8, CK18 and Kit, although in lower levels if compared with luminal carcinomas (Eroles *et al.*, 2012). Additionally, this type of tumor is usually associated with germ-line BRCA1 and BRCA2 mutations, high expression of DNA repair proteins, FOXM1 activation and presents a high rate of p53 mutations, leading to high genomic instability and focal amplifications (Vuong *et al.*, 2014).

Based on the immunophenotype profile, a variety of biomarkers have been proposed to define the basal-like subtype in mammary carcinoma (Nielsen *et al.*, 2004; Cheang *et al.*, 2008). The expression of one or more basal biomarkers, like high molecular weight basal cytokeratins (CK5/6, CK14 and CK17) or the EGFR, is the most commonly accepted method to identify the basal-like differentiation (Badve *et al.*, 2011). Therefore, basal-like subtype is characterized by the absence of the three major receptors of breast cancer, ER, PR and HER2 expressions, which led to the term of triple negative. However, TN tumors and basal-like tumors are not equivalent, once up to 30% of the TN do not express basal cytokeratins nor EGFR (Thike *et al.*, 2010). Consequently, the IHC panel used to defined the basal-like subtype includes the following five biomarkers: ER, PR, HER2, CK5/6 and EGFR (Nielsen *et al.*, 2004).

Based on the St Gallen International Expert Consensus guidelines, the basal-like subtype was defined as ER/PR and HER2 negative. This is justified due to the extensive overlap between triple negative and intrinsic basal-like subtype (Goldhirsch *et al.*, 2013).

Morphologically, the basal-like subtype is classified as infiltrating ductal carcinomas with no special type, although tubular, metaplastic and medullary carcinomas have already been associated with this subtype (Livasy *et al.*, 2006; Rakha *et al.*, 2008; Bosch *et al.*, 2010; Vuong *et al.*, 2014). They present high histologic tumor grade, large tumor size at diagnosis, marked cellular pleomorphism, high nuclear-cytoplasmic ratio, lack of tubule formation, high

mitotic index, frequent apoptotic cells, tumor necrosis, expanding margins and an evident stromal lymphocytic response (Livasy *et al.*, 2006; Rakha *et al.*, 2008; Bosch *et al.*, 2010).
Basal-like subtype is more prevalent at an early age, and is described as presenting higher prevalence among women of African origin (Bosch *et al.*, 2010; Weigelt *et al.*, 2010). Moreover, it is associated with an aggressive clinical history, higher frequency of lymph node metastasis, higher risk of distant metastasis and death within the first 3-5 years after the diagnosis, which has justified the association of basal-like subtype with poor outcome in breast cancer patients (Rakha *et al.*, 2008; Dreyer *et al.*, 2013).
The pattern of metastatic relapses is aggressive, with an increased propensity to metastize in visceral organs (such as lung, CNS and lymph nodes) and less likely to affect bone and liver (Rakha *et al.*, 2008; Eroles *et al.*, 2012).
Adding to this aggressive behaviour, there is currently no effective treatment available for the management of this subtype. Whereby, the identification of new therapeutic targets for the development of new treatment strategies is crucial (Dreyer *et al.*, 2013). One of the most promising strategies to treat this subtype of tumor is the use of poly-ADP ribose-polymerase-1 (PARP-1) inhibitors, like olaparib, iniparib or veliparib (Curigliano, 2012). PARP-1 is essential in DNA single strand breaks repair, whereby its inhibition plus an already defective DNA repair by BRCA1 mutation promotes the accumulation of breaks in double-stranded DNA and to apoptosis (Fong *et al.*, 2009).
Nowadays, the latest recommendation to treat patients with basal-like tumors is the use of anthracycline regimens (Goldhirsch *et al.*, 2013).

3.2.2.1.1. BRCA

In humans, breast cancer susceptibility 1 (BRCA1) and breast cancer susceptibility 2 (BRCA2) genes have been found to be mutated in a large number of early onset breast or ovarian cancers. Somatic loss of heterozygosity (LOH) is one of the genetic alterations associated with breast cancer initiation and progression (Miller *et al.*, 2003). In patients that carry germ line mutations in tumor suppressor genes such as BRCA1 or BRCA2, acquired loss-of-function mutations in the wild-type allele, will induce a deficient homologous-recombination DNA repair responsible for promoting carcinogenesis (Osorio *et al.*, 2002; Fong *et al.*, 2009).
Studies in humans have shown that breast cancer with BRCA1 mutations and less commonly BRCA2 mutations will predominantly display a triple negative basal-like immunophenotype. Actually, more than 75% of the breast tumors in women carrying a BRCA1 mutation display a triple-negative immunophenotype (Lakhani *et al.*, 2002; Foulkes *et al.*, 2003).
BRCA1 has a crucial role in normal mammary gland development and is necessary for the conversion of ER negative cells to ER positive. Moreover, it has been implicated in DNA

repair, transcriptional regulation and chromatin remodelling (Bosch *et al.*, 2010; Eroles *et al.*, 2012). Similarly, BRCA2 is implicated in DNA recombination and repair process and these two proteins are essential in the homologous recombination process. Therefore, the inactivation of these genes leads to the accumulation of errors and genetic instability, which benefits the development and growth of tumors (Fong *et al.*, 2009; Bosch *et al.*, 2010; Eroles *et al.*, 2012).

3.2.2.2. Normal-like

Normal-like subtype represents 5 to 10% of all breast carcinomas (Eroles *et al.*, 2012). This tumor is poorly characterized and its clinical significance is not well determined due to the few existing studies. Some researchers claim that normal-like subtype is a result of technical artefacts, like a contamination with normal tissue during the microarrays and yields doubts about their real existence (Weigelt *et al.*, 2010).

Similarly to basal-like subtype, the normal-like carcinomas belong to TN subgroup and lack expression of ER, HER2 and PR. However, they are negative for basal biomarkers, like CK 5/6 and EGFR. They express gene characteristics of adipose tissue, presenting an intermediate prognosis between luminal and basal-like tumors. In addition, these tumors will normaly not respond to adjuvant chemotherapy (Eroles *et al.*, 2012; Vuong *et al.*, 2014).

3.2.3. Claudin-low

After the initial molecular classification into subtypes of breast cancer, a new intrinsic subtype was identified by Herschkowitz *et al.*, 2007, designed “Claudin-low”. Claudin-low subtype has a predicted contribution of 12 to 14% of all breast cancers (Eroles *et al.*, 2012).

This subtype is characterized by the low expression of genes involved in tight junctions and intercellular adhesion, including claudins 3, 4, 7, occluding, cingulin and E-cadherin. Similarly to the basal-like subtype, claudin-low presents low expression of HER2 combined with low to absent luminal gene expression (Herschkowitz *et al.*, 2007). In contrast, there is a decrease in proliferative genes expression associated to an increase in the expression of genes involved in immune system response, cell communication, extracellular matrix, cell differentiation, cell migration and angiogenesis (Prat *et al.*, 2010). Overall, claudin low subtype is associated with the overexpression of epithelial-to-mesenchymal transition markers, immune response genes and presents cancer stem cell-like features (Prat *et al.*, 2010).

At protein level, claudin-low subtype is usually TN but also presents low to absent expression of E-cadherin and claudin 3 (Prat *et al.*, 2010). Even so, the concordance between claudin-low and TN is not 100%, and about 20% of the claudin-low subtypes are positive for hormone receptors (Eroles *et al.*, 2012).

The majority of claudin-low tumors present poor prognosis, invasive ductal carcinomas with high frequency of metaplastic and medullary differentiation, and display insufficient response to neoadjuvant chemotherapy (Prat *et al.*, 2010).

3.3. Other subtypes

Molecular apocrine subtype and interferon rich subtype are not as well described as other molecular profiles but have already been identified in several studies (Farmer *et al.*, 2005; Doane *et al.*, 2006; Teschendorff *et al.*, 2007; Banneau *et al.*, 2010; Colombo *et al.*, 2011; Vuong *et al.*, 2014).

The molecular apocrine subtype is characterized by the activation of androgen receptor (ADR) signalling and the ER-negative expression. Frequently this subtype displays HER2 gene amplification and germline PTEN mutations (Banneau *et al.*, 2010; Colombo *et al.*, 2011).

The molecular apocrine subtype is also associated with apocrine histological features such as abundant eosinophilic cytoplasm and prominent nucleoli in tumor cells (Vuong *et al.*, 2014). Based on a few number of studies, this molecular subtype seems to be associated with poor long-term survival (Farmer *et al.*, 2005). Nonetheless, the functional role of ADR in breast cancer is still poorly understood (Doane *et al.*, 2006).

The interferon-rich subtype is an ER-negative tumor and presents high expression of interferon-regulated genes, like STAT1 (Hu *et al.*, 2006). Its prognostic and clinical significance is still unclear but Teschendorff *et al.* (2007) indicate that this subtype presents a better prognosis when compared to the rest of ER-negative tumors. Hu *et al.* (2006) indicate a similar survival time to luminal B subtype.

3.4. Feline mammary tumors

After the discrimination of the different molecular profiles in breast cancer, some researchers applied this molecular classification to companion animals (Gama *et al.*, 2008; Sassi *et al.*, 2010; Brunetti *et al.*, 2013; Kim *et al.*, 2013; Beha *et al.*, 2014; Caliari *et al.*, 2014; Im *et al.*, 2014).

In FMC, two recent studies used an immunohistochemical panel in order to define five different molecular subtypes: luminal A, luminal B, HER2-positive, basal-like and normal-like tumors (Brunetti *et al.*, 2013; Beha *et al.*, 2014). Brunetti and colleagues (2013) concluded that luminal B subtype was the most common (52.4%, 11/21) within primary tumors, and that HER2-positive subtype was the most common in paired regional lymph nodes (76.2%, 16/21). Moreover, in the same study, researchers compared the molecular phenotypes of the two locations (primary tumors *versus* regional metastasis) and only 57.1% (12/21) of the animals presented a concordant classification. These results suggest a progressive loss of expression of the hormone receptors of the metastatic lesions. Constrasting to these results,

Wiese *et al.* (2013) and Caliari *et al.* (2014) concluded that triple negative subtype were the most common in FMC.
Wiese *et al.* (2013) also used a panel of immunohistochemical biomarkers to identify the triple negative basal-like subtype. In that study, no abnormality in BRCA1 and BRCA2 alleles was detected (n=5), but the triple negative subtype was the most common subtype (58.3%, 14/24).

4. Animal models

Cancer is a complex disease and requires different experimental systems for its study, from cell line cultures to animal models and finally, to clinical trials (Pinho *et al.*, 2012).
In this context, comparative oncology can integrate the study of naturally occurring cancers in animals into studies of human cancer biology and therapy. Several companion animals (pets) develop spontaneous neoplasias, which allow a unique form to investigate cancer biology and drug development. The most used species in comparative oncology are dogs and cats (Paoloni & Khanna, 2008).
Scientists listed several potential advantages of using companion animals with cancer for researcher's purposes, instead of the manipulated rodent models that are extensively used in oncology research (Porrello *et al.*, 2006; Airley, 2012). Besides the ethical issues concerning the use of laboratory animals, cat shares the same environment that humans. Consequently, they are subjected to the same carcinogens, unlike transgenic rodents. They also present a higher degree of genetic heterogeneity among cat population and like in humans, cancers occur naturally unlike experimental animal models, where an experimental induction takes place. Moreover, the time between the exposure to potential carcinogen and the development of the neoplasia is shorter than the human counterpart. Nevertheless, they share several biological similarities such as the size and physiology. These features allow the use of these animal models for pharmacokinetics and toxicological studies, especially the emetic toxicity (one of the major limitations in murine studies). In case of the therapeutic studies, the use of companion animals as models also presents advantages for the extrapolation of the doses range to humans. This is important for the subsequent phase I trials in humans. Surprisingly, the use of a similar animal model allows the decrease of the duration of phase I trials in humans and consequently reduces the morbidity and mortality associated to this experimental phase (de las Mulas & Reymundo, 2000; Porrello *et al.*, 2006; Paoloni & Khanna, 2008; Airley, 2012).
Other advantages of using companion animals in cancer research include the possibility to collect samples more easily (such as serum, urine or cerebrospinal fluid) due to the bigger size of these animals when compared to rodents. It also facilitates the surgical interventions and other procedures (Vail & MacEwen, 2000).

Another limitation of laboratory animals that can be overcome with the use of companion animals is the study of the interactions between tumor cell and the host tissue environment as well as the study of disease progression that potentially could be most similar to the human counterpart (Airley, 2012). They can be used to immunological studies, once dogs and cats have intact immune systems, contrasting to several rodent animal models. In addition, studies with dogs have demonstrated that tumor establishment and metastatic progression present similarities with the human counterpart (Khanna & Hunter, 2005).
Furthermore, companion species have a higher incidence of some types of cancer (osteosarcomas or non-Hodgkin's lymphomas) than humans (de las Mulas & Reymundo, 2000). And finally, the use of companion animals in oncology research can generate well defined clinical trials that can promote a reduction in cost of investigation in human clinical trials, and also better consent of the public and animal rights movements (Vail & MacEwen, 2000). The reduce number of established guidelines for oncological management in veterinary medicine allow the use of new and innovative treatment strategies on untreated cancer animals, as long as abuses are prevented, like unethical treatments or the deny of effective treatments to an animal in order for it to be included in a clinical trial (Withrow *et al.*, 2013). Generally, owners of companion animals usually are very committed and comply with the treatments, recheck visits and permission to perform necropsy is significantly more easy than in most human clinical trials (Vail & MacEwen, 2000).
Focusing on cats, the high prevalence of FMC and the high homology between human and cat genome (Pontius *et al.*, 2007; Sorenmo *et al.*, 2013) led to the interest of this species as a potential model for the study of women breast cancer, especially in the study of the HRs negative tumors. FMC are characterized for having low levels of ER and PR expression and thus are mainly hormonally independent. Also cats with mammary tumors respond to chemotherapy, especially to doxorubicin regimens, in contrast to canine mammary carcinomas (Vail & MacEwen, 2000; Wiese *et al.*, 2013).
The main drawback of the use of companion animals relies on the relative lack of species-specific investigational tools, like reagents, DNA probes and libraries, monoclonal antibodies species-specific and canine and feline recombinant products (Vail & MacEwen, 2000; Withrow *et al.*, 2013). However, some research groups are currently developing molecular technologies for both species (Vail & MacEwen, 2000).
In summary, the use of companion animals as animal models could provide advances in oncology research benefiting both humans and animals, expanding the understanding of the biology of disease but also creating possibilities to the development of new therapeutic strategies that can improve the morbidity and mortality of these diseases.

5. Aims

Our group is particularly focused on the diagnostic and clinical relevance of FMC with overexpression of HER2 protein. Considering the scarce literature available for the study of FMC, the main purpose of this thesis was to contribute for the clinical and molecular characterization of cat mammary gland tumors in order to identify potential prognostic and predictive factors, with special interest in HER2 protein.
According to this, the core objectives are as follows:

- The aim of the study presented in Chapter I was the optimization of *f*HER2 protein and gene detection using IHC and FISH techniques, respectively. Since literature present few and inconsistent results for HER2 protein determination, the objective was to identify the more accurate protocol to evaluate HER2 protein and gene expression and determine its incidence in FMC as well as if the gene amplification phenomenon is conserved between human and feline species.
- In the Chapter II, the main purpose of our group was to investigate the prognostic value of Ki-67 index. As for HER2, the literature existent present inconsistent results. Considering the importance of this proliferation biomarker in breast cancer and it possible relationship with HER2 protein, the objectives of this study were to determine the ideal cut-off value of Ki-67 in FMC, their association with several clinicopathological features (including HER2) and determine its prognostic value.
- The study presented in Chapter III aimed to characterize the clinical and pathological features of the different molecular subtypes in cat, using a broad accepted IHC-based classification established by Sta. Gallen's International Expert Consensus panel for human breast cancer.
- In the Chapter IV we focus in metastatic disease progression of FMC, once is the major issue in veterinary and human oncology. Thus, the main objective of this study was to determine the protein status and the molecular subtype of metastatic lesions and compare its concordance with the primary tumors. Overall, we wanted to characterize the metastatic disease in cats with FMC, once studies in this field are very scarce and outdated.
- Finally, in Chapter V our aim was to detect the extracellular domain fragment of *f*HER2 in feline serum (serum *f*HER2-ECD). This can assume great importance in Veterinary Oncology, especially as a complement of IHC for *f*HER2 detection and as a tool for monitoring the progression of the disease. The objective of this study was to accurately quantify the sHER2 levels in cats and to evaluate the usefulness of measuring sHER2 levels in the diagnosis of FMC overexpressing *f*HER2.

The overarching goal of the research herein presented is to increase our understanding regarding the molecular characterisation of FMC, especially regarding the HER2 protein and its clinical implications as a prognostic tool and as a potential therapeutic target. Besides the improvement of the knowledge in veterinary oncology, these studies also pretend to support the use of cats as a suitable animal model for the study of breast cancer.

EXPERIMENTAL WORK

EXPERIMENTAL WORK

Chapter I – Feline HER2 Protein Expression Levels and Gene Status in Feline Mammary Carcinoma: Optimization of Immunohistochemistry (IHC) and In Situ Hybridization (ISH) Techniques

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1. Abstract

Human epidermal growth factor receptor (HER2) is a tumor biomarker that when overexpressed and/or amplified is associated with a poor prognosis for women with breast cancer. This specific tumor subtype is eligible for a specific immunotherapy that increases survival period. However, in feline oncology, only a few studies have been performed on molecular characterisation of feline (*f*HER2) in feline mammary carcinoma (FMC), and the available data are inconsistent. In this study, *f*HER2 protein levels and gene status in FMC were evaluated by immunohistochemistry and in situ hybridization. After being optimised, these techniques revealed that *f*HER2 is overexpressed in 33% of FMC cases, although *f*HER2 and *f*TOP2A gene amplification could not be observed. Our results support the possibility of using FMC as a natural model for comparative oncology. Additional data obtained may also improve the diagnostics, and consequently the treatment, of this type of tumor in veterinary medicine.

Keywords: feline mammary carcinomas (FMC), feline human epidermal growth factor receptor-2 gene homolog (*f*HER2), feline topoisomerase 2 alpha gene homolog (*f*TOP2A), gene amplification, protein overexpression, immunohistochemistry (IHC), in situ hybridization (ISH)

2. Introduction

Feline mammary tumors are the third most common malignancy showing a short overall survival period of <12 months (Sorenmo *et al.*, 2013). These tumors, usually classified as carcinomas, display clinical features and an aggressive phenotype similar to those reported in breast cancer in women overexpressing the human epidermal growth factor receptor-2 (HER2). This transmembrane glycoprotein belongs to the epidermal growth factor receptor family and is involved in a variety of molecular pathways associated with tumor growth and progression. In humans, the accurate assessment of HER2 has become critical for chemoimmunotherapy planning (Vollan & Caldas, 2011). For this, clinical oncologists evaluate HER2 protein levels by immunohistochemistry (IHC), and classify tumors as HER2 positive (HER2+) or HER2 negative (HER2–). Equivocal results, classified as 2+ by IHC, lead to the determination of HER2 gene status by the *in situ* hybridization (ISH) technique (Rampaul *et al.*, 2002; Rosa *et al.*, 2009; Susini *et al.*, 2010). Recent data reveal that about 20–30% of human breast cancer patients display HER2 overexpression, and 80% of them show HER2 gene amplification (Wolff *et al.*, 2007), prompting a therapy based on a humanized anti-HER2 monoclonal antibody (Trastuzumab; Roche, Basel, Switzerland), which improves survival rates (Stebbing *et al.*, 2000; Wolff *et al.*, 2007). Furthermore, topoisomerase II alpha gene (TOP2A) is also amplified in 40% of HER2+ breast cancer patients, encouraging a HER2/TOP2A combined inhibitory therapy (Tanner *et al.*, 2006; Fountzilas *et al.*, 2012; Wang *et al.*, 2012).

Recent studies have shown a strong association between feline HER2 (*f*HER2) oncogene homolog overexpression and mammary carcinogenesis, although its biological role remains unclear. Further studies on *f*HER2 protein overexpression incidence are needed, as divergences of the reported data in feline mammary carcinomas (FMC) vary from 5 to 92% (De Maria *et al.*, 2005; Millanta *et al.*, 2005a; Winston *et al.*, 2005; Ordás *et al.*, 2007; Rasotto *et al.*, 2011).

This study aims to optimise *f*HER2 immunodetection in feline tumor samples using different antigen retrieval methods and testing five commercial antibodies. In addition, we assess *f*HER2 and *f*TOP2A gene status in FMC-HER2+ samples using six commercial hybridization DNA probes and verify whether the gene amplification phenomenon is conserved between human and feline species. To achieve comparable data, the American Society of Clinical Oncology (ASCO) guidelines were followed. In particular, sample fixation time and immunohistochemical and fluorescence *in situ* hybridization (FISH) HER2 scoring criteria were used. In our opinion, the conflicting results published by several authors about FMC-*f*HER2+ incidence can be explained by the absence of standardized guidelines for *f*HER2 assessment in evaluation of human breast cancer samples.

This study provides new insights on FMC-HER2+ molecular characterisation, confirming FMC as a suitable model for comparative oncology studies (Porrello *et al.*, 2004, Porrello *et al.*, 2006).

3. Material and Methods

3.1. Tumor Sample Collection and Histology

Thirty formalin-fixed, paraffin-embedded (FFPE) FMC tissue samples previously fixed for <72h were obtained from the Veterinary Pathology Diagnostic Service archives (Veterinary Medicine Faculty, Technical University of Lisbon). A human HER2+ breast cancer sample collected at the Medicine Faculty (University of Lisbon) was used as a positive control. For histological classification, 4 µm sections were stained with hematoxylin and eosin (H&E) and tumors were classified according to the World Health Organization (WHO) criteria (Misdorp *et al.*, 1999) being scored from I to III in the malignancy grade (Elston & Ellis, 1998).

3.2. Immunohistochemical Studies

Primary Antibodies

The following commercial anti-HER2 antibodies were tested in feline FFPE sections: rabbit polyclonal (A0485; Dako, Glostrup, Denmark); rabbit monoclonal (4B5; Ventana, Tucson, AZ, USA); mouse monoclonal (TAB250; Invitrogen, Carlsbad, CA, USA); rabbit monoclonal (SP3); and a mouse monoclonal (CB11) both from Zytomed (Berlin, Germany).

Immunohistochemical Technique

For each tumor, a selected area was chosen to guarantee the presence of tumor cells while avoiding tissue necrosis. Immunohistochemical protocols were performed using 4-µm-thick sections, mounted on microscope slides (Star-frost®, Knittel Glaser, Bielefeld, Germany), and dried at 60ºC for one hour. Each slide was deparaffinized and rehydrated in a graded alcohol series, followed by an antigen retrieval step in which the tissue sample was incubated with citrate buffer solution ($NaCH_3COO$, pH 6.0) in a pressure chamber (2 atm, 2 min) or in a water bath (95ºC, 30 or 60 min) as described in Table 5. In addition, other antigen retrieval conditions were tested to improve TAB250 antibody specificity: microwave cycling with citrate buffer solution, pH 6.0 (850 W, 15 min), microwave cycling with Tris-EDTA buffer solution, pH 9.0 (900 W, 5 min plus 600 W, 15 min) or enzymatic digestion with Proteinase K (Zymed) for 10 min. After blocking endogenous peroxidase activity (peroxide-block solution; Zytomed) for 10 min, each tissue slide was then incubated with a different anti-HER2 primary antibody at room temperature for 1 h. After two PBS washes, the primary antibodies were detected by a 30 min incubation of the HRP-polymer-enhanced IHC detection system (HER2*easy* kit IHC; Zytomed) with 3,3'-diaminobenzidin-tetrahydrochlorid (DAB; Dako), before counterstaining with Mayer’s hematoxylin.

A human HER2+ breast cancer slide previously scored as 3+ by IHC (Table 6) was used as the positive control, whereas a human HER2– breast cancer slide scored as 0 (Table 6) was used as the negative control. All the FMC samples were also incubated with either a purified rabbit polyclonal IgG (ab27472; Abcam, Cambridge, UK) or a purified mouse polyclonal IgG (ab37355; Abcam) instead of the anti-HER2 primary antibodies, in order to discard species cross-reactivity.

Table 5. Summary of the IHC protocols used for *f*HER2 detection.

Primary antibody			
Clone	**Dilution(s)**	**Incubation time (min)**	Antigen retrieval method
A0485	1:250 1:300	60 min	Boiling water bath (95ºC) for 30 min or 60 min with citrate buffer solution (pH 6.0) *or* Pressure chamber at 2 atm during 2 min with citrate buffer solution (pH 6.0)
CB11	RTU	60 min	
4B5	RTU	60 min	
SP3	1:100	60 min	
TAB250	1:50 1:250	60 min	Boiling water bath (95ºC) for 30 min or 60 min with citrate buffer solution (pH 6.0) *or* Pressure chamber at 2atm for 2 min with citrate buffer solution (pH 6.0) *or* Microwave (850 W for 15 min) in citrate buffer solution (pH 6.0) *or* Microwave (900 W for 5 min plus 600 W for 15 min) in Tris-EDTA solution (pH 9.0) *or* Proteinase K for 10 min

IHC, Immunohistochemistry; RTU, ready-to-use

fHER2 Interpretation Criteria

Tumor *f*HER2 positivity was determined on the basis of the maximum area of staining intensity according to the DAKO/ASCO guidelines (Table 6), in which *f*HER2 overexpression is defined as a strong membranous staining in more than 10% of the neoplastic cells. FMC samples scored as 0 or 1+ were considered negative, whereas those scored as 2+ or 3+ were considered HER2+. Cytoplasmic staining was considered as nonspecific.

Table 6. HER2 IHC scoring criteria (HercepTest interpretation manual, DAKO).

Score	Interpretation
0	No staining.
1+	Weak, incomplete membranous staining in any proportion of tumor cells.
2+	Complete membrane staining that is either no uniform or weak in intensity but with obvious circumferential distribution in at least 10% of cells.
3+	Uniform intense membrane staining of at least 10% of invasive tumor cells.

All slides were submitted to blind scoring by two independent pathologists, and discordant interpretation was further debated and settled using a multiviewer microscope.

3.3. ISH Studies

DNA Probes

The *f*HER2 and *f*TOP2A gene status evaluation in FMC tissue samples was performed by FISH, chromogenic in situ hybridization (CISH), and silver in situ hybridization (SISH). FISH was tested using: a Vysis TOP2A/HER2/CEP17 FISH Probe (Vysis; Downers Grove, USA), a ZytoLight SPEC HER2/TOP2A/CEN17 Triple Color Probe (Zytovision, Bremerhaven, Germany), and a HER2 FISH pharmDx kit (Dako). CISH was done with a SPOT-Light HER2 DNA Probe (Zymed) and a ZytoDot SPEC HER2 Probe (Zytovision). Finally, SISH was performed with an Inform HER2 Dual ISH DNA Probe Cocktail (Ventana).

ISH Technique

Optimization of ISH was performed using four FMC-HER2+ cases scored as 3+ by IHC. For this purpose, 23 different protocols were tested to determine *f*HER2/*f*TOP2A gene status variability under different pretreatment conditions, enzymatic digestions, commercial probes, stringent wash, and incubation periods to different immunodetection systems. After optimization, all FMC-HER2+ samples were investigated using the following protocol: tissue sections were dewaxed in two 10-min changes of xylene, rehydrated through two 3-min changes of 100% ethanol, one 3-min change of 95% ethanol, one 3-min step of 70% ethanol, and immersed for 3 min in distilled water. The slides were placed in wash buffer solution (K5599; DAKO) in two 3 min changes before the pretreatment, which was performed by microwave cycling (180 W) in four 5-min exposures using 2-[*N*-morpholino]ethanesulphonic acid buffer (MES buffer; Dako). After cooling for 15 min, the slides were placed in wash buffer solution (K5599; Dako) in two 3-min changes, and then incubated in a pepsin proteolytic solution (K5599; Dako) at 37ºC for 3–5 min. Then the tissue sections were briefly washed with wash buffer solution for two 3-min changes, immersed in distilled water, dehydrated in ascending alcohol concentrations, and air-dried. A measure of 10–20 µL of the probe was applied per slide according to the size of the tissue and covered with a sealed coverslip. Tissue sections were placed in a Dako Hybridizer (model S2451), which was used to perform DNA denaturation (75ºC for 10 min) and hybridization (overnight at 37ºC). Thereafter, slides were washed for 5 min in post-hybridization buffer (2xSSC/0.3% NP-40 detergent) at room temperature to remove the coverslips and placed in post-hybridization buffer at 37ºC for 5 min. After a brief rinse in wash buffer solution, slides were dehydrated, air-dried (protected from direct light), counterstained with DAPI/Vectashield mounting medium (Vector Laboratories, Peterborough, UK), and covered with a coverslip for observation.

Interpretation Criteria

*f*HER2 and *f*TOP2A gene amplification in FMC tissue samples were evaluated using a confocal laser point-scanning microscope (LSM 710Axio Observer; Carl Zeiss Inc., Jena, Germany). Settings for the individual fluorophores were as follows: 4',6-diamidino-2-phenylindole (DAPI and centromeric region of human chromosome 17), excitation at 418 nm and recording at 445–480 nm; fluorescein isothiocyanate (FITC for HER2 gene detection),excitation at 503 nm and recording at 520–550 nm; rhodamine (TOP2A gene detection), excitation at 547 nm and recording at 560–600 nm. Tissue areas with nonoptimal enzymatic digestion (poor nuclear resolution or persistent autofluorescence) were rejected and slides were blind-scored by two microscopists. Tumor samples with an average of >6 HER2 gene copies per cell or a ratio between HER2/CEP17 (or TOP2A/ CEP17) above 2.2 were considered HER2+, according to the ASCO guidelines for human samples (Table 7). Data sets were acquired by Zen software (Blue Edition, Carl Zeiss) and images were subsequently processed with Adobe Photoshop CS6 software (Adobe Systems Inc., San Jose, CA, USA).

Table 7. HER2 and TOP2A gene amplification assessment (ASCO guidelines, Tanner *et al.*, 2006; Wolff *et al.*, 2007).

Average counting of HER2 or TOP2A copy number for cell nucleus (HER2/CEP17 or TOP2A/CEP17 ratio)	Interpretation
< 4 (<1.8)	Negative for HER2 or TOP2A gene amplification
4.0-6.0 (1.8-2.2)	Equivocal range of values (need of additional cell count or retest)
>6.0 (>2.2)	Positive for HER2 or TOP2A gene amplification

4. Results

4.1. *f*HER2 Overexpression Evaluation in FMC

From the five antibodies tested, CB11, 4B5, and A0485 revealed specific and strong cellular membrane labeling in some FMC samples (Figure. 12a and 12b). Neither TAB250 nor SP3 antibodies showed any species cross-reactivity. From all of the different protocols tested, the best results were obtained whenever extreme antigen retrieval conditions were performed (Table 8). Indeed, when the A0485 polyclonal antibody was associated with the longer AgR method (water bath at 95ºC for 60 min), the best *f*HER2 protein recognition was achieved (33% of the FMC cases were classified as *f*HER2+). CB11 and 4B5 antibodies gave similar results, particularly when AgR was performed in the pressure chamber (8 and 6 FMC-HER2+ representing 26.7 and 20%, respectively).As expected, the human positive control showed an intense and uniform membrane labeling (scored as 3+, Figure 12c), whereas the human negative control did not show any staining (Figure 12d). The feline negative control slides demonstrated the specificity of the membrane staining as no labeling could be detected.

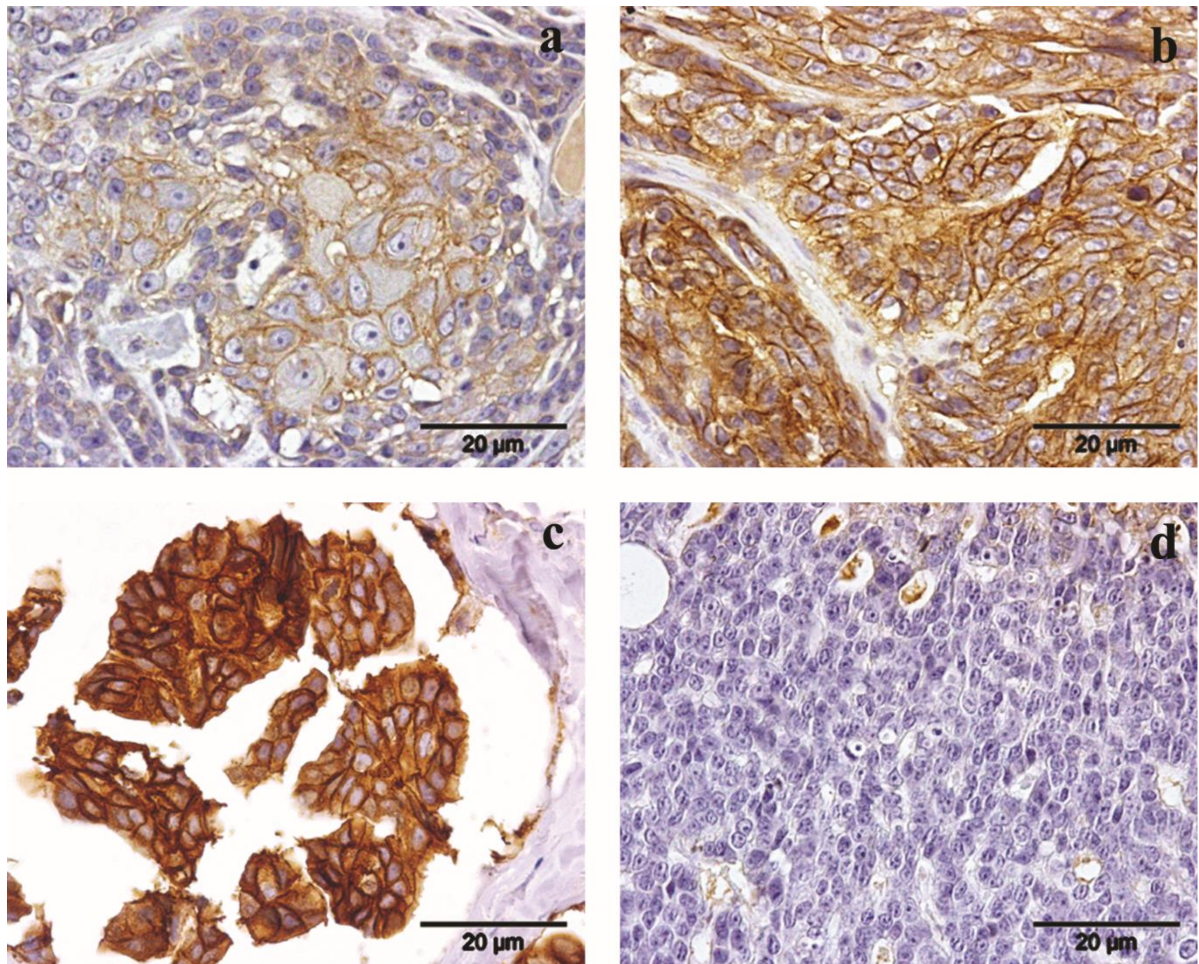

Figure 12. Evaluation of *f*HER2 by IHC.

(a) *f*HER2 overexpression in a squamous mammary carcinoma scored as 2+; **(b)** FMC-HER2+ cribiform classified as 3+; **(c)** human positive control showing an intense and uniform membrane labeling scored as 3+; **(d)** human HER2—breast cancer used as negative control. All samples were counterstained with Mayer's hematoxylin.

4.2. ISH Study

The ten FMC samples scored as 2+ or 3+ by IHC were subjected to ISH analysis in order to verify whether *f*HER2 overexpression is correlated with *f*HER2 gene amplification. After the protocol optimization, neither *f*HER2 nor *f*TOP2A presented gene amplification, as only one or two signals for each gene could be detected per nucleus (Figure 13a). Attending to the species sequence specificity, the centromeric region of the feline chromosome E1 could not be detected (Figure 13a) by using DNA probes recognizing the centromere of the human homologous chromosome 17 (Figure 13b).

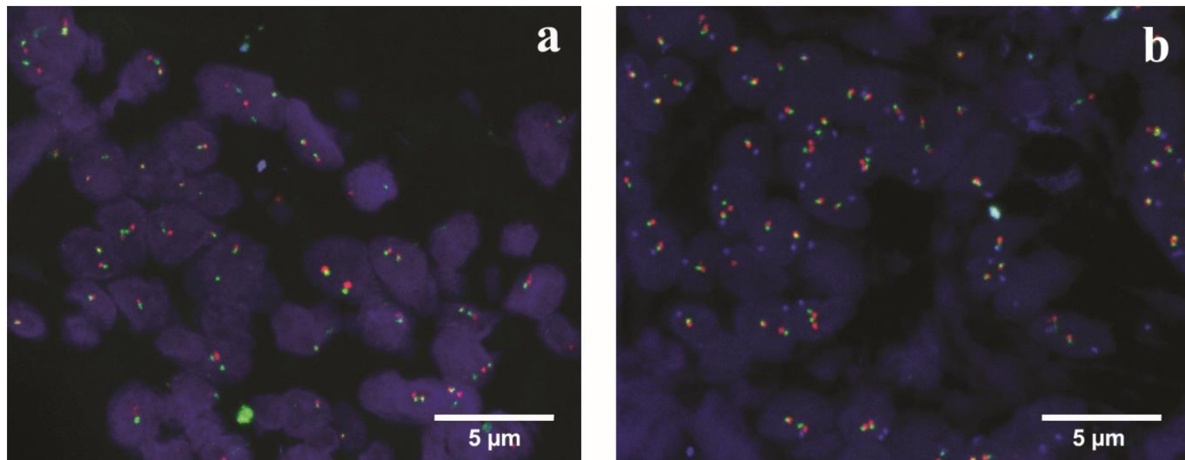

Figure 13. *f*HER2 and *f*TOP2A genes were not amplified in FMC-HER2+.

(a) *f*HER2 and *f*TOP2A detection by FISH with *f*HER2 gene (green signal) and *f*TOP2A gene (red signal) were not amplified in FMC samples; **(b)** human HER2—breast cancer showing HER2/TOP2A non-amplified genes and the alpha satellite centromeric region of the chromosome 17 (blue signal).

5. Discussion

In the last decade, efforts have been made to standardize the different molecular techniques to assess HER2 status in humans, in order to achieve reliable and reproducible results (Ménard *et al.*, 2000; Gancberg *et al.*, 2002; Bilous *et al.*, 2003; Wolff *et al.*, 2007; Rosa *et al.*, 2009). Currently, IHC and FISH are the two main techniques routinely performed in human oncology, with well-defined criteria that include pre-analytic conditions (type and time of fixation), analytic indicators (scoring definitions), and post-analytic conditions (interpretation criteria) (Bilous *et al.*,2003; Wolff *et al.*, 2007).

The results obtained in this study show that *f*HER2 is overexpressed in 33.3% of FMC cases, whenever the A0485 antibody was associated with the longer antigen retrieval protocol (water bath at 95ºC for 60 min), similar to results previously published (De Maria *et al.*, 2005; Ordás *et al.*, 2007) and to the incidence reported in women (Wolff *et al.*, 2007).

In the course of this study, the results varied either with the use of different primary antibodies or protocol conditions, and this outcome is congruent with previously published data (one study indicated 5.5%, whereas another referred 92.5% of *f*HER2 positive cases). In fact, CB11 and 4B5 antibodies demonstrated an inferior sensitivity to identify *f*HER2, when compared with A0485 antibody (with 26.7 and 20% of positive cases, respectively), whereas SP3 and TAB250 antibodies did not show any labeling. The absence of labeling with these antibodies, which recognize the HER2 extracellular domain, could be due to the deleterious fixation effects of formaldehyde or differences between HER2 human extracellular domain and *f*HER2,which have a homology of 94%, whereas A0485, CB11, and 4B5 recognize the intracellular domain (O'Malley *et al.*, 2001; Sidoni *et al.*, 2006; Ricardo *et al.*, 2007; Nassar *et al.*, 2009). Altogether, these discrepancies indicate the importance of the technique standardization in order to obtain accurate results.

Table 8. Results of IHC using A0485, CB11 and 4B5 as primary antibodies.

*f*HER2 score		0	1+	2+	3+	TOTAL
Antibody	**AgR method**					
A0485	WB1	16 (53.3%)	5 (16.7%)	8 (26.7%)	1 (3.3%)	30 (100%)
	WB2	15 (50%)	5 (16.7%)	6 (20%)	4 (13.3%)	
	PC	6 (20%)	16 (53.3%)	5 (16.7%)	3 (10%)	
CB11	WB1	24 (80%)	6 (20%)	0 (0%)	0 (0%)	
	WB2	18 (60%)	10 (33.3%)	2 (6.7%)	0 (0%)	
	PC	9 (30%)	13 (43.3%)	6 (20%)	2 (6.7%)	
4B5	WB1	23 (76.7%)	6 (20%)	1 (3.3%)	0 (0%)	
	WB2	15 (50%)	10 (33.3%)	5 (16.7%)	0 (0%)	
	PC	16 (53.3%)	8 (26.7%)	4 (13.3%)	2 (6.7%)	

IHC, immunohistochemistry; AgR, antigen retrieval; WB1, water bath at 95ºC with citrate buffer solution for 30 min; WB2, water bath at 95ºC with citrate buffer solution for 60 min; PC, pressure chamber at 2 atm with citrate buffer solution for 2 min.

The analysis of *f*HER2 and *f*TOP2A gene status was only possible by using one of five commercial probe mixtures, probably because *f*HER2 and the human HER2 genes share 88% sequence homology (De Maria *et al.*, 2005; Santos *et al.*, 2012). None of the FMC-HER2+ tumor samples displayed *f*HER2 and/or *f*TOP2A gene amplification, denoting that the oncogenic mechanism responsible for *f*HER2 overexpression is different from the one reported in human HER2+ breast tumor (Ménard *et al.*, 2000; Cianciulli *et al.*, 2002).

Nevertheless, our data are in agreement with the results reported by the unique study that evaluated *f*HER2 gene status in FMC (Ordás *et al.*, 2007).

In addition, *f*TOP2A gene amplification was not detected in FMC-HER2+ samples, in accordance with what has been described in human HER2+ breast cancer cases. The TOP2A gene is known to be rarely amplified when the HER2 gene is not (Bhargava *et al.*, 2005; Oakman *et al.*, 2009). The absence of *f*TOP2A gene amplification raises questions about the beneficial effects of using doxorubicin-based chemotherapy in FMC (McNeill *et al.*, 2009; Sorenmo *et al.*, 2013).

In feline oncology, mammary tumors are usually highly aggressive, growing rapidly and metastasize to regional lymph nodes and the lungs, sharing several clinical features with the human breast cancer HER2+ subtype. This enhances the possibility of using cats as a breast cancer model (De Maria *et al.*, 2005).

Table 9. Optimised protocol steps for HER2 detection in feline mammary carcinomas by IHC and FISH techniques.

Technique	Antibody/DNA probe recommend	Optimised protocol Steps
IHC	A0485 (dilution 1:300)	AgR method with boiling WB
FISH	ZytoLight SPEC HER2/TOP2A/CEN17 Triple Color Probe	Pretreatment: MW with MES buffer (4x5 min) Pepsin proteolytic solution (37ºC 3-5 min) Posthybridization: Buffer (SSC/NP-40) 5 min.

IHC, immunohistochemistry; AgR, antigen retrieval; WB, water bath at 95ºC with citrate buffer solution for 60 min; FISH, Fluorescent *In Situ* Hybridization; MW, Microwave cycling (180W); MES, 2-[N-morpholino]ethanesulphonic acid buffer; SSC/NP-40, 2x SSC/0.3% NP-40 detergent.

6. Conclusions

In summary, our results indicate that *f*HER2 protein and gene status evaluation should be performed following the optimised protocols described in Table 9. The improved IHC protocol revealed that *f*HER2 is overexpressed in about 33% of FMC cases, highlighting the relevance of immunostaining standardization in veterinary diagnosis. The evaluation of *f*HER2 and *f*TOP2A gene status demonstrated that neither of these genes is amplified in FMC-HER2+ using the FISH technique, raising doubts about the efficiency of anthracycline-based chemotherapy regimens in cat. In addition, our results strongly suggest the possibility of using FMC-HER2+ as a suitable cancer model to study human HER2+ breast carcinomas without gene amplification.

Chapter II – Ki-67 as prognostic factor in feline mammary carcinoma – what is the optimal cut-off value?

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1. Abstract

Ki-67 is a nuclear protein and a proliferation marker frequently used in establishing the prognosis for breast cancer patients. To investigate the prognostic value of the Ki-67 proliferation index in female cats with mammary carcinoma, a prospective study was conducted with 96 animals. The Ki-67 index of primary tumors (n=96) was initially determined, and whenever possible, the Ki-67 index of regional lymph node metastasis (n=38) and distant metastasis (n=16) was also estimated. The optimal cut-off value for the Ki-67 index was determined by univariate and multivariate analysis. Ki-67 indices ≥14% were detected in 72.9% (70 of 96) of the tumors. Tumors with a Ki-67 index ≥14% were significantly associated with large size (P=.022), poor differentiation (P=.009), presence of necrotic areas (P=.008), estrogen receptor-negative status (P<.0001), *f*HER2-negative status (P=.003), and shorter overall survival (P=.012). Moreover, Ki-67 expression in the primary tumor was strongly and positively correlated with both regional metastasis (P<.0001; r=0.83) and distant metastasis (P<.0001; r=0.83), and was significantly higher in distant metastases when compared with the primary tumor (P=.0009). A similar correlation was also observed between regional and distant metastasis (P<.0001; r=0.75). On the basis of the above results, the authors propose the adoption of the 14% value as the optimal cut-off for Ki-67 to identify tumors with high risk of disease progression.

Keywords: feline mammary carcinoma; Ki-67 proliferation index; prognostic factor; cut-off value

2. Introduction

Feline mammary carcinomas (FMCs) are among the most prevalent tumors in cats, with an incidence that can reach 40% of all tumor cases in this species (Millanta *et al.*, 2002a; Vascellari *et al.*, 2009; Sorenmo *et al.*, 2013). Contrary to those of humans and dogs, 85% to 93% of feline mammary tumors are considered malignant, and the most common histotypes are the tubular, papillary, solid, and cribriform carcinomas (Sorenmo *et al.*, 2013). Feline mammary tumors may metastasize to the regional lymph nodes, lungs, and liver, as well as other organs. Although some studies correlate age, tumor size, presence of regional metastasis, and malignancy grade with overall survival (OS), still scarce information is available regarding the proliferation index of FMC (Weijer & Hart, 1983; MacEwen *et al.*, 1984; Seixas *et al.*, 2011). Based on this scenario, additional prognostic and predictive factors may provide useful insights into tumor biology to achieve a more efficient therapy and a better follow-up of the affected female cats.

Ki-67 is a protein found only in growing, dividing cells. It is expressed in all cell cycle phases, except in the resting phase (G0). With an intranuclear localization, the expression levels of Ki-67 are low during the G1 and S phases, rapidly increase during the G2 phase, and reach a peak in mitosis. This well-defined expression pattern makes the Ki-67 antigen a good proliferation marker, very useful and reliable in the prognosis of human breast cancer (Yerushalmi *et al.*, 2010; Dowsett *et al.*, 2011). Several studies of women demonstrated that high Ki-67 expression is a poor prognostic indicator of 5-year recurrence-free survival in breast cancer patients (DeCensi *et al.*, 2011; Dowsett *et al.*, 2011; Tanei *et al.*, 2011; Luporsi *et al.*, 2012; Denkert *et al.*, 2013; Ono *et al.*, 2015), being also used for chemotherapy response prediction (DeCensi *et al.*, 2011; Dowsett *et al.*, 2011; Luporsi *et al.*, 2012). However, different Ki-67 cut-off values and tumor heterogeneity require an adaptation of the scoring system, with cut-off values varying from 3.5% to 34% (Harry *et al.*, 2007; Dowsett *et al.*, 2011; Denkert *et al.*, 2013; Pathmanathan *et al.*, 2013). To overcome this problem, a large study recently recommended the standardization of a Ki-67 cut-off point at 14% in breast cancer to improve prognosis (Cheang *et al.*, 2009).

In veterinary medicine, although the Ki-67 index has been investigated as a prognostic factor in FMC, few data are available on how the Ki-67 index correlates with lower survival (Millanta *et al.*, 2002a; Preziosi *et al.*, 2002; Seixas *et al.*, 2011). Furthermore, the evaluation of cell proliferation by Ki-67 immunostaining has not been subject to standardization of protocols. The lack of clear indication on a cut-off level explains why no values have been validated for FMC (Castagnaro *et al.*, 1998c; Preziosi *et al.*, 2002; Pereira *et al.*, 2004; Seixas *et al.*, 2011).

The main goal of the present prospective study is to validate the prognostic value of the Ki-67 index in FMC. First, the Ki-67 index of 96 primary mammary tumors was determined and compared with corresponding lymph node and distant metastasis. Second, the ideal cut-off

value was calculated by evaluating a set of threshold values based on univariate and multivariate survival analysis. Finally, the association between the Ki-67 index and 19 other clinicopathologic features of cats with mammary carcinoma was evaluated.

3. Materials and Methods

3.1. Animal population

The studied population consisted of 96 female cats with mammary tumors admitted to the Small Animal Hospital of the Faculty of Veterinary Medicine of the University of Lisbon from June 2011 to December 2013. Animals were followed for at least a month to be included in survival analysis, and the last follow-up was in May 2014. For each animal, the following clinical data were recorded: age, breed, reproductive status (intact vs spayed), administration of progestogens, number and location of tumors, tumor stage (TNM system), treatment prescribed (none, mastectomy, mastectomy combined with chemotherapy), and OS (Sorenmo *et al.*, 2013). Additionally, the presence of microscopic regional lymph node metastasis was evaluated in a majority of cases (86 cats). A full postmortem examination was performed in 18 cats, and metastatic disease was confirmed histologically in 16 of those animals.

All mammary and respective metastatic lesions excised during surgery from 89 cats or during necropsy (7 cats) were fixed in 10% buffered formalin for 24 to 48 hours and embedded in paraffin. The histologic specimens were evaluated following the World Health Organization classification system (Misdorp *et al.*, 1999). The degree of malignancy was determined by the assessment of 3 morphologic features (tubule formation, nuclear pleomorphism, and number of mitosis) and scored from I (well-differentiated tumor) to III (poorly differentiated tumor), according to the Elston and Ellis grading system (Elston & Ellis, 1998). Detailed information was also collected about tumor size, histopathologic classification, malignancy grade, presence of necrotic areas, lymphatic invasion by neoplastic cells, lymphocytic infiltration, and skin ulceration.

3.2. Immunohistochemistry (IHC)

The Ki-67 proliferation index, the status of estrogen receptor (ER) and progesterone receptor (PR), and the expression of feline epidermal growth factor receptor type 2 (*f*HER2) and cytokeratin 5/6 (CK 5/6) were evaluated in all mammary carcinomas (*n*=213), lymph node metastasis (*n*=59), and distant metastasis (*n*=69) collected during surgery or necropsy.

A representative area of each lesion (diameter, 0.6 cm) was selected and used to prepare 5 serial 3-μm sections, which were later attached to SuperFrost Plus microscope slides (Thermo Scientific, Rockford, IL, USA). Tissue sections were then dried at 60ºC for 1 hour and deparaffinized. The antigen retrieval was performed by boiling samples for 2 minute in a sodium citrate buffer solution (0.01M $NaCH_3COO$, pH 6.0) using a pressure cooker (2 atm)

(Burrai *et al.*, 2010; Soares *et al.*, 2013a). For PR immunodetection, antigen retrieval was achieved with the same sodium citrate buffer in a water bath for 60 minutes at 95ºC. For CK 5/6, samples were microwaved at 900 W for 15 minutes in Tris-EDTA buffer (pH 9.0) (Brunetti *et al.*, 2013).

Immunohistochemical analysis was performed with the primary antibodies summarized in Supplemental Table 1, and staining was achieved with a modified streptavidin–peroxidase conjugate method (Novolink MaxPolymer Detection System, Leica Biosystems, Wetzlar, Germany). Finally, tissue sections were counterstained with Mayer's hematoxylin (Merck, NJ, USA).

Feline tonsil was used as positive control for Ki-67 index, while normal skin was used as a positive control for CK5/6 expression. Feline mammary samples with known positive and negative ER, PR, and *f*HER2 status were also employed as positive controls.

Immunohistochemistry staining was scored by 2 independent observers, and discordant interpretations were settled using a multiviewer microscope. Tumor cells were considered Ki-67 positive in the presence of brown nuclear staining of granular or diffuse type (Cheang *et al.*, 2009; Pereira *et al.*, 2004; Yerushalmi *et al.*, 2010; Dowsett *et al.*, 2011). The Ki-67 proliferation index was determined by assessing the percentage of positively staining tumor cell nuclei in 1000 tumor cells. For each lesion, 5 to 6 images were randomly taken with an Olympus DP25 camera (Pennsylvania, USA) on an Olympus BX51 light microscope and analyzed using Image J (Open Source Software, version 1.46r; National Institutes of Health, Bethesda, MD, USA).

ER and PR expression was evaluated in tumor tissues using the Allred score system (Suppl. Table 2), where a score ≥3 is considered positive (Harvey *et al.*, 1999; Mohsin *et al.*, 2004; Hammond *et al.*, 2010). *f*HER2 staining intensity was evaluated by using the Food and Drug Administration–approved scoring system (Hammond *et al.*, 2010; Wolff *et al.*, 2013), in which tumors with immunohistochemistry scores of 3+ (uniform and intense membrane staining of at least 10% of tumor cells) or 2+ (complete membrane staining, not uniform or weak in intensity but with obvious circumferential membrane distribution in at least 10% of cells) were considered HER2 positive (Millanta *et al.*, 2005a; Burrai *et al.*, 2010; Brunetti *et al.*, 2013; Santos *et al.*, 2013; Soares *et al.*, 2013a). Finally, tumors were considered CK 5/6 positive when >1% of cells were immunoreactive (Adamczyk etal., 2012; Brunetti *et al.*, 2013; Joensuu *et al.*, 2013).

3.3. Determination of the optimal cut-off value for Ki-67 index

Whenever a cat exhibited multiple mammary tumors, the lesion with higher risk of malignancy was selected for further statistical analysis based on tumor size and malignancy grade, which both have been associated with worse prognosis in FMC (Weijer & Hart, 1983; MacEwen *et al.*, 1984; Seixas *et al.*, 2011).

After testing for normality, the paired *t* test was applied to make a comparison between the Ki-67 index of primary tumors and regional metastasis, between the Ki-67 index of primary tumors and distant metastasis, and between the Ki-67 index of regional and distant metastasis. Correlation analyzes were performed by using the Pearson correlation test. When multiple metastases were present (regional and/or distant), the lesions with higher Ki-67 expression were chosen for statistical analysis.

To determine the optimal cut-off value for Ki-67 in FMC, an univariate survival analysis was performed testing the following range of values, also used in human breast cancer patients: 5%, 10%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 25%, 30%, and 35% (Dowsett *et al.*, 2011; Denkert *et al.*, 2013; Pathmanathan *et al.*, 2013). Regarding the survival analysis, OS was defined as the time, in months, between the initial diagnosis and death or the date of the last follow-up for surviving cats (censored observations). Only deaths attributed to mammary carcinoma progression were considered. In this study, 2 deaths were caused by other diseases (censored observations), both belonging to the group of cats with high Ki-67 indexes: one was caused by renal failure, and the other animal was euthanized because owners could not afford the proposed surgery. Among the total of the cats in the study (*n*=96), 7 were excluded for survival analysis, once they were followed for less than a month. OS curves were estimated using the Kaplan-Meier method and compared with the log-rank test.

The cut-off values that showed significance were subjected to multivariate analysis using the Cox proportional hazard model. The hazard ratio was calculated with a 95% confidence interval (95% CI).

After the establishment of the Ki-67 cut-off, the positive predictive value (PPV) and negative predictive value (NPV) were calculated for predicting death and survival after 1 and 2 years after the surgery. For this analysis, only the animals subjected to surgery were considered, with those that also received chemotherapy being excluded.

The Fisher exact test was used to assess the association between Ki-67 expression and 19 clinicopathologic features: age, breed, reproductive status, previous administration of progestogens, tumor number, location and size, stage of disease, histopathologic classification, malignancy grade, presence of necrotic areas, lymphatic invasion, lymphocytic infiltration, cutaneous ulceration, regional lymph node metastasis, receptor status (ER, PR, fHER2), and CK 5/6 expression. For each significant association, odds ratio (OR) was calculated with a 95% CI.

The association between Ki-67 labeling index and tumor size was analyzed after dividing the population into 3 subgroups, according to the tumor size, using the levels established in the TNM classification system (<2, 2–3, and >3 cm). To evaluate the effect of age, the population was split into 3 subgroups: <8, 8–12, and >12 years old.

Quantitative data were processed and analyzed with SPSS 21.0 (IBM, New York, NY, USA), and a 2-tailed $P<.05$ was considered statistically significant.

4. Results

4.1. Animal population data

Ninety-six female cats with mammary carcinoma were followed up, and their clinicopathologic features are summarized in Supplemental Table 3. The mean ±SD age of the animals was 11.49 ±2.85 years (range, 5–19 years). The majority of the cats were subjected to surgery (n=89), including unilateral mastectomy in 71 (79.8%), regional mastectomy in 11 (12.4%), and bilateral mastectomy in 7 (7.8%). Eight mastectomized cats received anthracycline-based chemotherapy after surgery (doxorubicin, 25 mg/m2, intravenously, every 3 weeks for 5 cycles [Sorenmo *et al.*, 2013]).

In this study, 213 mammary carcinomas were collected from the 96 queens. In the 60 animals that showed >1 tumor, the most malignant lesion was chosen (see Materials and Methods). The mean size of the tumors was 2.71 ±1.5 cm (range, 0.5–7 cm).

By the end of the study, 48 animals had died or were euthanized due to metastatic progression of the disease, and for those animals, the median OS was 10.5 months (mean, 12.2 months).

4.2. Ki-67 index of primary tumor is strongly and positively correlated with Ki-67 index of regional and distant metastasis

The immunohistochemical analysis of the Ki-67 expression revealed a moderate to strong nuclear staining of tumor cells (Figure 14a), especially in distant metastases (Figure 14b). The mean Ki-67 index was 26% ±1.8% in primary tumors (range, 0.2%–85.8%; median, 21.4%), rising to 30.1% ±3.1% in regional lymph node metastases (range, 3.5%–78.8%; median, 26.4%, n=38). Distant metastases (n=16) showed a mean Ki-67 index of 48.2% ±5.1% (range, 13.3%–92.1%; median, 44.6%).

A strong and positive correlation was found between the Ki-67 index of the primary tumor and regional metastasis ($P<.0001$; r=0.83, n=38), between the Ki-67 index of the primary tumor and distant metastasis ($P<.0001$; r=0.83, n=16), and between the Ki-67 index of regional and distant metastasis (P=.0052; r=0.75, n=16).

In addition, the Ki-67 index of primary tumors was significantly lower when compared to the Ki-67 index of distant metastasis (P=.0009, n=16). Differences between regional and distant metastasis approached significance (P=.08, n=16), with distant lesions showing higher Ki-67 scores. Finally, primary tumors and regional metastasis presented distinct Ki-67 scores, which were not statistically significant (P=.186, n=38).

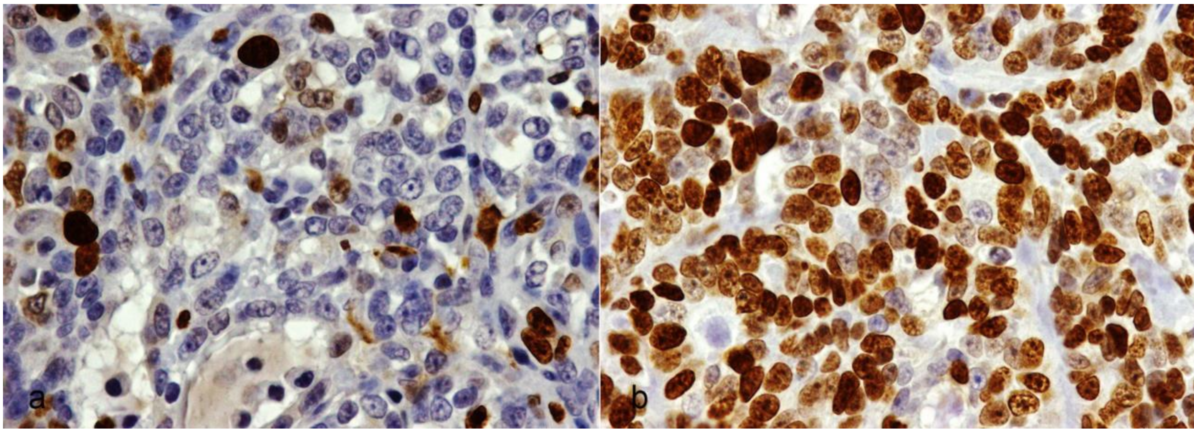

Figure 14. Cribriform carcinoma, mammary gland, cat. Nuclear Ki-67 staining.

1a – Primary tumor with a Ki-67 index of 13.7%; **1b** – Lung metastasis with a Ki-67 index of 88.6%. Immunohistochemistry for Ki-67.

4.3. The optimal cut-off value of Ki-67 index is 14% in feline mammary carcinoma

To determine the optimal cut-off point for the Ki-67 index, a univariate survival analysis was performed using different cut-off values (5%, 10%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20 %, 25 %, 30 %, and 35%). Statistically significant differences in OS curves were observed only for the cut-off of 14% (*P*=.012), 16% (*P*=.019), and 17% (*P*=.027; Table 10, Figure 15). Further multivariate analysis showed that a 14% cut-off value could better distinguish cats with highly malignant mammary carcinomas from those with low malignant potential. In addition, only a cut-off value of 14% was identified to be a Ki-67 independent prognostic indicator of OS (*P*=.027), showing a hazard ratio of 2.4 (Table 10).

Table 10. Univariate and Multivariate analysis of Ki-67 proliferation index

Univariate: Ki-67	Animals, n	OS Time (months) Mean ± SD	95% CI	*p*
Cut-off of 14%				0.012
Low, <14%	23	33.32 ±3.61	26.2, 40.4	
High, ≥14%	66	22.55 ±2.80	17.1, 28.0	
Cut-off of 16%				0.019
Low, <16%	31	30.31 ±3.26	23.9, 36.7	
High, ≥16%	58	22.28 ±3.01	16.4, 28.2	
Cut-off of 17%				0.027
Low, <17%	33	31.77 ±3.58	24.8, 38.8	
High, ≥17%	56	22.17 ±3.18	15.9, 28.4	
Multivariate: Ki-67		**HR**	**95% CI**	***p***
Cut-off of 14%		2.4	1.1, 5.1	0.027
Cut-off of 16%		-	-	0.436
Cut-off of 17%		-	-	0.115

Abbreviations: CI, Confidence interval; HR, Hazard ratio; OS, overall survival.

The majority of the animals that presented low Ki-67 values were alive by the end of the follow-up period (69.6%, 16 of 23), while only 37.9% (25 of 66) of cats with higher Ki-67

values (≥15%) were alive. Moreover, most of the cats that died had a primary tumor with a Ki-67 index ≥14% (85.4%, 41 of 48), and only 7 presented low Ki-67 values (14.6%, 7 of 48).

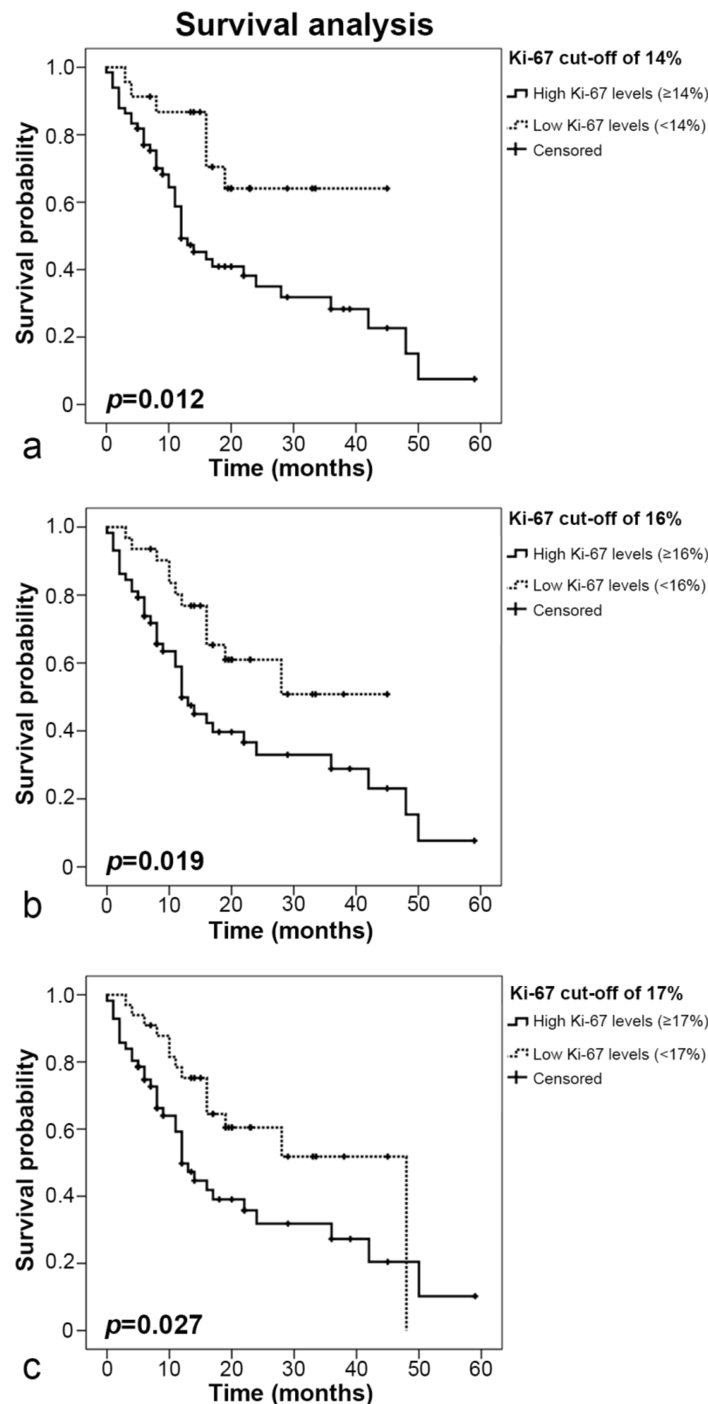

Figure 15. Kaplan-Meier overall survival (OS) curves using different cut-off values of Ki-67.

Overall survival curves with a Ki-67 cut-off value of 14% **(a)**, 16% **(b)** and 17% **(c)**. In all scenarios, cats with feline mammary carcinoma showing a Ki-67 index below the cut-off value, survived significantly longer than those with higher Ki-67 indexes.

4.4. Animals with high Ki-67 index (>14%) have higher probability of dying within the 2 years after surgery

With the 14% as a threshold value, the PPV and NPV were calculated for predicting death or survival at 1 and 2 years after surgery. For 1 year after surgery, the PPV for predicting death was 50%, and the NPV was 81.8%. For 2 years of survival after surgery, the PPV was 80%, and the NPV was 27.3%.

4.5. Ki-67 index ≥14% is significantly associated with unfavorable features

Of the 96 cats with mammary carcinoma, 70 showed mammary tumors with a Ki-67 index ≥14% (72.9%), while 26 cats showed lower Ki-67 indices (27.1%). The majority of the cats with mammary carcinoma presenting with regional lymph node metastasis had a Ki-67 index ≥14% (78.9%, 30 of 38). Of 16 metastatic disease cases, 15 (93.8%) also had distant metastasis with a Ki-67 index ≥14%.

Moreover, primary mammary carcinomas that showed a Ki-67 index ≥14% were significantly associated with large size (*P*=.022; OR, 4.4; 95% CI: 1.12, 21.65), poor differentiation (grade III, *P*=.009), presence of necrotic areas (*P*=.008; OR, 3.7; 95% CI: 1.25, 11.13), negative ER status (*P*<.0001; OR, 7.12; 95% CI: 2.38, 22.66), and low *f*HER2 expression (*P*=.003; OR, 4.31; 95% CI: 1.47, 12.98; Supplemental Table 4). In contrast, no significant statistical association was found between Ki-67 index ≥14% and age, breed, reproductive status, previous administration of progestogens, tumor location, multiple mammary tumors, lymph node metastasis, disease stage, histopathologic classification, lymphatic invasion by neoplastic cells, lymphocytic infiltration, skin ulceration, and PR or CK5/6 status of the primary tumor (Suppl. Table 4).

5. Discussion

Until now, the World Health Organization histopathologic classification has been extensively used to establish the prognosis in cats and dogs with mammary tumors (Elston & Ellis, 1998; Misdorp *et al.*, 1999; Sorenmo *et al.*, 2013). Although tumor cell proliferation has emerged as an important diagnostic and prognostic tool in human breast cancer patients (Perou *et al.*, 2000; Cheang *et al.*, 2009; Dowsett *et al.*, 2011; Fasching *et al.*, 2011; Keam *et al.*, 2011;Park *et al.*, 2012), no standard cut-off point for the Ki-67 index is known in FMCs (Seixas *et al.*, 2011). Recently, some studies assessed Ki-67 expression in feline mammary tumors and showed that malignant lesions exhibited higher Ki-67 levels that benign lesions (Castagnaro *et al.*, 1998c; Millanta *et al.*, 2002a; Preziosi *et al.*, 2002; Burrai *et al.*, 2010; Rasotto *et al.*, 2011; Seixas *et al.*, 2011). In the present study, we demonstrated that the Ki-67 labeling index in the primary tumor reveals a strong and positive correlation with the Ki-67 index of local and distant metastasis. Moreover, and besides the small sample (*n*=16), the metastatic lesions had significantly higher Ki-67 indices than the primary lesions (means of

48.2% vs 26%), corroborating the association between Ki-67 overexpression in the primary tumor and aggressive tumor behavior.

Employing the methodology used in human clinical studies (Ono *et al.*, 2015; Pathmanathan *et al.*, 2013), the optimal cut-off value for Ki-67 was determined. The Ki-67 cut-off of 14% was considered as the optimal value to predict clinical outcome of FMC. Indeed, cats whose primary tumors showed a Ki-67 index ≥14% displayed a 2.4-fold increased risk of tumor-related death, in comparison with cats having mammary tumors that exhibited a lower Ki-67 index (Table 10). Most primary tumors (72.9%) and metastatic lesions (93.8%) showed a Ki-67 index ≥14%, with the corresponding cats presenting significantly shorter survival. In fact, the majority of cats that died from mammary cancer disease during the follow-up showed a primary tumor with a Ki-67 index ≥14% (41 of 48, 85.4%).

The Ki-67 index also demonstrated a strong predictive value, with a high proportion of cats with low levels of Ki-67 alive after 1 year of the surgery (NPV=81.8%). Moreover, high Ki-67 index levels also demonstrated an important predictive value, as the probability of the cat dying within the 2 years after surgery was 80% (i.e., PPV=80%).

In parallel, we also observed a strong association between Ki-67 levels and several clinicopathologic features associated with shorter OS, such as size of the tumor (Weijer & Hart, 1983; MacEwen *et al.*, 1994), malignancy grade (Seixas *et al.*, 2011), presence of necrotic areas (Pereira *et al.*, 2004; Seixas *et al.*, 2011), and low ER expression (Millanta *et al.*, 2006a), which reinforces the relevance of this marker for the clinical follow-up of FMC.

In human breast cancer, a positive ER status is associated with low cancer mortality (Hammond *et al.*, 2010; Deyarmin *et al.*, 2013). Our results corroborate this scenario in FMC. Regarding HER2 tumor status, its overexpression is associated with worse prognosis in breast cancer (Ménard *et al.*, 2000; Wolff *et al.*, 2013); however, its role in FMC is still controversial. It was reported that overexpression of *f*HER2 is associated with short OS periods (Millanta *et al.*, 2005a). However, a recent report claimed that *f*HER2 overexpression is associated with feline tumors that show non malignant features (Santos *et al.*, 2013). Our findings support these results, since mammary tumors with Ki-67 levels ≥14% were significantly associated with a negative *f*HER2 status, indicating that *f*HER2 overexpression could be associated with a better clinical outcome.

Recent studies also showed that Ki-67 immunohistochemistry measurements can significantly predict breast cancer outcome in women treated with anthracycline-based chemotherapy (Ohno *et al.*, 2013; Rocca *et al.*, 2014; Tan *et al.*, 2014). Anthracyclines are a class of antitumor drugs (e.g., doxorubicin) that inhibit a family of enzymes essential for cell division (topoisomerases type II), making these drugs a primary choice in the treatment of tumors with high growth rates, being also used to treat a variety of malignancies in cats, including mammary carcinoma (Sorenmo *et al.*, 2013). To the best of our knowledge, there are no studies showing that chemotherapy improves survival in cats with mammary

carcinomas showing a high Ki-67 index. Unfortunately, in our study, only 8 cats (8.3%) with mammary carcinoma were treated with doxorubicin after surgery, not allowing us to assess the value of Ki-67 in predicting the benefit of anthracycline-based chemotherapy.

In conclusion, our study demonstrated that the Ki-67 index can be used as a prognostic biomarker in cats with mammary carcinoma—specifically, it showed that Ki-67 values ≥14% are associated with lower OS and with other aggressive clinicopathologic features. All results presented above suggest that a Ki-67 cut-off of 14% can be regarded as a useful tool to identify animal patients with worse prognosis.

6. Supplemental material

Supplemental Table 1. Immunohistochemical protocols used for Ki-67, ER, PR, *f*HER2 and CK 5/6 detection

Antigen	Primary antibody			Antigen retrieval method
	Clone	**Dilution**	**Incubation period and temperature**	
Ki-67	Polyclonal[a]	1:500	60 min, RT	Sodium citrate buffer (0.01M $NaCH_3COO$, pH 6.0) in a pressure cooker at 2 atm, for 2 min
HER2	CB11[b]	1:200	O/N, 4ºC	
ER	6F11[a]	1:100	O/N, 4ºC	
PR	1E2[c]	RTU	O/N, 4ºC	Sodium citrate buffer (0.01M $NaCH_3COO$, pH 6.0) in a water bath, 95ºC for 60 min
CK 5/6	D5/16 B4[a]	RTU	O/N, 4ºC	Tris-EDTA buffer (pH 9.0) in a microwave (900W for 15 min)

Abbreviations: O/N, overnight; RT, room temperature; RTU, ready-to-use.
[a] Thermo Scientific, Rockford, USA; [b] Invitrogen, Carlsbad, USA; [c] Ventana, Tucson, USA.

Supplemental Table 2. Allred score system for ER and PR evaluation

% of positive staining tumor cells		**Average staining intensity**	
Score	Interpretation	Score	Interpretation
0	No staining	0	None
1	<1%	1	Weak
2	1-10%	2	Average
3	10-33%	3	Strong
4	33-66%		
5	>66%		

Allred score (0-8) = score from the % of positive staining tumor cells (0-5) plus average staining intensity score (0-3)

Supplemental Table 3. Clinicopathological features of the 96 female cats with mammary carcinoma

Features	Number of animals (%)
Age	
< 8 years	16 (17.2%)
8-12 years	44 (47.3%)
> 12 years	33 (35.5%)
Unknown[a]	3
Breed	
Undetermined	77 (80.2%)
Siamese	9 (9.4%)
Persian	6 (6.3%)
Norwegian Forest Cat	3 (3.1%)
Blue Russian	1 (1.0%)
Spayed	
No	48 (51.1%)
Yes	46 (48.9%)
Unknown[a]	2
Progestogens	
No	29 (39.7%)
Yes	44 (60.3%)
Unknown[a]	23
Treatment	
None	7 (7.3%)
Mastectomy	81 (84.4%)
MCh	8 (8.3%)
Multiple mammary tumors	
No	36 (37.5%)
Yes	60 (62.5%)
Lymph node status	
Negative	48 (55.8%)
Positive	38 (44.2%)
Unknown[a]	10
Disease stage (TMN)	
I	22 (22.9%)
II	17 (17.7%)
III	42 (43.8%)
IV	15 (15.6%)
Tumor location	
M1	16 (16.9%)
M2	21 (22.1%)
M3	37 (38.9%)
M4	21 (22.1%)
Unknown[a]	1
Size	
<2 cm	31 (32.3%)
2-3 cm	36 (37.5%)
>3 cm	29 (30.2%)
HP classification	
Tubulopapillary carcinoma	56 (58.4%)
Solid carcinoma	17 (17.7%)
Cribriform carcinoma	13 (13.5%)
Mucinous carcinoma	9 (9.4%)
Squamous cell carcinoma	1 (1.0%)
Malignancy grade	
I	3 (3.2%)
II	16 (16.8%)
III	76 (80.0%)
Unknown[a]	1

Necrosis	
No	25 (26.0%)
Yes	71 (74.0%)
Lymphatic invasion	
No	79 (82.3%)
Yes	17 (17.7%)
Lymphocytic infiltration	
No	27 (28.1%)
Yes	69 (71.9%)
Ulceration	
No	83 (86.5%)
Yes	13 (13.5%)
ER status	
Negative	70 (72.9%)
Positive	26 (27.1%)
PR status	
Negative	55 (57.3%)
Positive	41 (42.7%)
***f*HER2 status**	
Negative	70 (72.9%)
Positive	26 (27.1%)
CK 5/6 status	
Negative	45 (48.4%)
Positive	48 (51.6%)
Unknown[a]	3

Abbreviations: HP, Histopathological; M, mammary gland; MCh, mastectomy plus chemotherapy
[a] Not considered when calculating the percentage.

Supplemental Table 4. Association between the Ki-67 index (cut-off value of 14%) and clinicopathological features

Features	Total (n=96)	Ki-67 index Low (<14%)	Ki-67 index High (≥14%)	*p*
Age				0.36
< 8 years	16	7	9	
8-12 years	44	11	33	
> 12 years	33	8	25	
Unknown[a]	3	0	3	
Breed				0.42
Undeterminated	77	21	56	
Siamese	9	3	6	
Persian	6	1	5	
NFC	3	1	2	
Blue Russian	1	0	1	
Spayed				0.21
No	48	15	33	
Yes	46	10	36	
Unknown[a]	2	1	1	
Progestogens				0.49
No	29	7	22	
Yes	44	12	32	
Unknown[a]	23	7	16	
LNS				0.21
Negative	48	15	33	
Positive	38	8	30	
Unknown[a]	10	3	7	
Disease Stage (TNM)				0.65
I	22	7	15	
II	17	6	11	
III	42	9	33	
IV	15	4	11	
Multiple mammary tumors				0.54
No	36	10	26	
Yes	60	16	44	
Tumor location				0.37
M1	16	2	14	
M2	21	6	15	
M3	37	11	26	
M4	21	7	14	
Unknown[a]	1	0	1	
Size				0.022
<2 cm	31	13	18	
2-3 cm	36	9	27	
>3 cm	29	4	25	
HP classification				0.28
Tubulopapillary carcinoma	56	17	39	
Solid carcinoma	17	6	11	
Cribriform carcinoma	13	3	10	
Mucinous carcinoma	9	0	9	
Squamous cell carcinoma	1	0	1	
Malignancy grade				0.009
I	3	3	0	
II	16	6	10	
III	76	17	59	
Unknown[a]	1	0	1	
Necrosis				0.008
No	25	12	13	
Yes	71	14	57	

Dermal lymphatic invasion				0.098
No	79	24	55	
Yes	17	2	15	
Lymphocytic infiltration				0.45
No	27	8	19	
Yes	69	18	51	
Ulceration				0.51
No	83	23	60	
Yes	13	3	10	
ER status				<0.001
Negative	70	11	59	
Positive	26	15	11	
PR status				0.58
Negative	55	15	40	
Positive	41	11	30	
***f*HER2 status**				0.003
Negative	70	13	57	
Positive	26	13	13	
Ck 5/6 status				0.42
Negative	45	15	30	
Positive	48	11	37	
Unknown[a]	3	0	3	

Abbreviations: HP, Histopathological classification; LNS, Lymph node status; M, mammary gland; NFC, Norwegian Forest Cat;
[a]Not considered in the statistical analysis.

Chapter III – Molecular based subtyping of feline mammary carcinomas and clinicopathological characterisation

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1. Abstract

Molecular classification of feline mammary carcinomas (FMC) from which specific behavioural patterns may be estimated has potential applications in veterinary clinical practice and in comparative oncology. In this perspective, the main goal of this study was to characterize both the clinical and the pathological features of the different molecular phenotypes found in a population of FMC (n=102), using the broadly accepted IHC-based classification established by St Gallen International Expert Consensus panel.

The luminal B/HER2-negative subtype was the most common (29.4%, 30/102) followed by luminal B/HER2-positive subtype (19.6%, 20/102), triple negative basal-like (16.7%, 17/102), luminal A (14.7%, 15/102), triple negative normal-like (12.7%, 13/102) and finally, HER2-positive subtype (6.9%, 7/102). Luminal A subtype was significantly associated with smaller tumors (p=0.024) and with well differentiated ones (p<0.001), contrasting with the triple negative basal-like subtype, that was associated with larger and poorly differentiated tumors (p<0.001), and with the presence of necrotic areas in the tumor lesion (p=0.003). In the survival analysis, cats with Luminal A subtype presented the highest survival time (mean OS=943.6 days) and animals with triple negative basal-like subtype exhibited the lowest survival time (OS mean=368.9 days). Moreover, two thirds (64%, 32/50) of the queens with multiple primary tumors showed different molecular subtypes in each carcinoma, revealing that all independent lesions should be analyzed in order to improve the clinical management of animals.

Finally, the similarities between the subtypes of feline mammary tumors and human breast cancer, reveal that feline can be a valuable model for comparative studies.

Keywords: feline; mammary carcinoma; immunohistochemistry; molecular subtype; luminal B; triple negative

2. Introduction

Feline mammary carcinomas have a deserved reputation of highly malignant behavior with most tumor types being considered similarly as bad news as far as prognosis is considered (Sorenmo *et al.*, 2013). However, the considerable variety of histological types and clinical outcomes up rise the concern that there may be some variations that need to be exploited as therapeutic approach may vary from surgical excision and nothing else to combinations of this with chemotherapy. The gain in discriminating between various types of prognosis may be not just beneficial for the female cats and their owners but also for the characterisation of an animal population, readily available, that has not been conveniently exploited in translational studies in cancerology. It has long been proven that feline mammary tumors have much more similarities with the human mammary tumors, than the same pathology in laboratory animals (de las Mulas *et al.*, 2000; De Maria *et al.*, 2005; Sorenmo *et al.*, 2013).

It is generally accepted that early detection and more effective treatments are key factors that explain longer survival times in human cases of mammary carcinoma (Edwards *et al.*, 2014). More effective treatments have benefited from advances in tumor classification evolving from systems based upon molecular and immunophenotypic markers. The first molecular classification for breast cancer was proposed by Perou and colleagues (2000), which divided breast tumors in four groups (luminal-like, basal-like, HER2+ and normal-like), according to their gene expression analysis (Perou *et al.*, 2000). The prohibitive cost of this multigenic assay, led to the development of alternatives, such as the evaluation of biomarkers using immunohistochemistry (IHC) analysis, which was suggested as a surrogate for gene expression profiling (Park *et al.*, 2012; Ribelles *et al.*, 2013). Recently, the St Gallen International Expert Consensus panel proposed a IHC-based classification that establishes six biologically distinct breast cancer subtypes: luminal A, luminal B/HER2-negative, luminal B/HER2-positive, HER2-positive (non-luminal), triple negative basal-like and triple negative normal-like (Goldhirsch *et al.*, 2013).

The luminal A is the most common subtype and the one with better prognosis. It is characterized by the overexpression of estrogen and/or progesterone receptor (ER and/or PR), HER2-negative status and low Ki-67 index (Eroles *et al.*, 2012; Goldhirsch *et al.*, 2013). Luminal B breast tumors display a more aggressive behavior than luminal A and are divided in two subtypes: the luminal B/HER2-negative which show positive staining for ER and/or PR, negative expression for HER2 and high Ki-67 index levels, and the luminal B/HER2-positive subtype that shows ER and/or PR and HER2 overexpression (Cheang *et al.*, 2009; Goldhirsch *et al.*, 2013). Patients with luminal tumors usually benefit from endocrine therapies. Within the non-luminal subtypes, the HER2-positive subtype is characterized by the HER2 overexpression in the absence of hormone receptors (ER and PR). This subtype is associated to a poor prognosis, but fortunately, specific anti-HER2 therapies have improved the survival rate of patients (Reis-Filho & Pusztai, 2011; Curigliano, 2012; Eroles *et al.*,

2012). Finally, the triple negative tumors show the worst prognosis of all breast cancer subtypes and are characterized by the lack of ER, PR and HER2 expression.

They are classified in basal-like and normal-like tumors, based upon the cytokeratin expression (CK 5/6, 14 and 17), that is positive in the basal-like tumors (Sørlie *et al.*, 2001; Gusterson *et al.*, 2005; Eerola *et al.*, 2008; Bosch *et al.*, 2010).

In Veterinary Medicine, some investigation has been conducted to find biomarkers that could improve the prognosis accuracy in cats with mammary carcinomas (Millanta *et al.*, 2002a; Sarli *et al.*, 2003; Millanta *et al.*, 2005a; Millanta *et al.*, 2006a; Millanta *et al.*, 2006b; Burrai *et al.*, 2010; Seixas *et al.*, 2011; Islam *et al.*, 2012; Peñafield-Verdu *et al.*, 2012; Soares *et al.*, 2016b). This effort is important since feline malignant mammary tumors are very common in cats, representing the third most common tumor in this species. They are predominantly malignant (85% to 95%) and clinically very aggressive (Weijer & Hart, 1983; MacEwen *et al.*, 1984; Sorenmo *et al.*, 2013). Recently, three studies have used a panel of markers to immunophenotype feline mammary carcinomas (FMC) (Brunetti *et al.*, 2013; Soares *et al.*, 2013b; Wiese *et al.*, 2013). However, the small number of tumor samples and the use of different classifications led to contradictory conclusions. In this study, we aimed to overcome this difficulty clinically characterizing the different subtypes identified in a large population of female cats presenting mammary carcinomas (n=102), using the IHC-based classification established by St Gallen International Expert Consensus panel (Goldhirsch *et al.*, 2013). Significant statistical associations between cancer subtypes and 19 clinicopathological features were evaluated, and a univariate analysis of overall survival (OS) and disease free survival (DFS) was performed.

3. Material and Methods

3.1. Study population

A total of 102 female cats with mammary carcinoma were enrolled in this prospective study from September 2009 to January 2014. Animals were presented at the Teaching Hospital of the Faculty of Veterinary Medicine, University of Lisbon (FVM-ULisboa), and the owners gave permission to collect samples from their pets and to use the animal's clinical data. All mammary tumors were surgically obtained after mastectomy, except for 9 cases that were collected at necropsy. Tumor samples were collected in accordance with the EU Directive 2010/63/EU and research was approved by the Ethics Committee of the FVM-ULisboa.

The following clinical and pathological features were evaluated and recorded: age, breed, reproductive status at the time of the surgery (intact versus spayed), previous administration of progestogens for oestrus control, number, location and size of tumor lesions, treatment performed (none, surgery, surgery combined with chemotherapy), extension of the surgery (lumpectomy, unilateral mastectomy or bilateral mastectomy), stage of the disease (TNM system, [Sorenmo *et al.*, 2013]), disease free survival (DFS) and overall survival (OS).

3.2. Tumor histological classification

Mammary tumors were fixed in 10% buffered formalin during 24 to 48 hours and were processed for routine histological examination. All tumors were classified according to the World Health Organization (WHO) classification system (Misdorp *et al.*, 1999). The malignancy grade was scored from I to III using the Elston & Ellis scoring system (Elston & Ellis, 1998) and the presence of necrotic areas within the lesions, lymphatic invasion by neoplastic cells, lymphocytic infiltration and cutaneous ulceration was recorded. In 93 cases, the regional lymph nodes were also collected and analyzed.

3.3. Immunohistochemistry

For each primary tumor, a specific area was selected (6 mm in diameter), avoiding the necrotic and the non tumor areas, sections were obtained and immunohistochemical staining was performed for detection of the following proteins: ER, PR, feline homologue of HER2 (*f*HER2), CK 5/6 and Ki-67. IHC protocols and score interpretation were performed as previously described (Soares *et al.*, 2013a; Soares *et al.*, 2013b; Soares *et al.*, 2015; Soares *et al.*, 2016b).

Classification of the staining results was made according to the Allred Score guidelines for interpretation of ER and PR staining (Table 11) and HER2 was interpreted according to the American Society of Clinical Oncology (ASCO) guidelines which criteria are summarized in Table 12 (Harvey *et al.*, 1999; Hammond *et al.*, 2010; Park *et al.*, 2012; Wolff *et al.*, 2013).

Table 11. Allred Score guidelines for ER and PR staining

Score for percentage of positive tumor cells		**Score for average intensity of staining**	
Score	Interpretation	Score	Interpretation
0	No staining	0	None
1	<1%	1	Weak
2	1-10%	2	Average
3	10-33%	3	Strong
4	33-66%		
5	>66%		
Allred score (0-8) = The Σ of both scores			

A tumor was considered positive for ER and PR when presenting a score >2 (Hammond *et al.*, 2010; Soares *et al.*, 2015; Soares *et al.*, 2016b); for *f*HER2 when achieving the score 2+ or 3+ (De Maria *et al.*, 2005; Millanta *et al.*, 2005a; Soares *et al.*, 2013a); and for CK 5/6 status, when revealing cytoplasmic and/or membrane labeling of 1% of the tumor cells (Adamczyk *et al.*, 2012; Brunetti *et al.*, 2013; Soares *et al.*, 2015). For Ki-67, a tumor was considered highly proliferative when presenting a Ki-67 index ≥14% (Soares *et al.*, 2016b).

Table 12. *f*HER2 immunohistochemistry scoring criteria

Score	Interpretation
0	No staining
1+	Weak, incomplete membrane staining in any proportion of tumor cells
2+	Complete membrane staining that is either no uniform or weak in intensity but with obvious circumferential distribution in at least 10% of cells
3+	Uniform intense membrane staining of at least 10% of invasive tumor cells

For molecular classification of the FMC the St Gallen International Expert Consensus panel was applied, using five biomarkers (ER, PR, HER2, Ki-67 and CK 5/6), as resumed in Table 13 (Goldhirsch *et al.*, 2013; Ribelles *et al.*, 2013; Ferguson *et al.*, 2013; Caldarella *et al.*, 2013; Soares *et al.*, 2015).

Table 13. Immunohistochemical definitions of the molecular subtypes in FMC

		Luminal B			Triple negative	
	Luminal A	**Luminal B /HER2-**	**Luminal B /HER2+**	**HER2-positive**	**Basal-like**	**Normal-like**
ER	ER+ and/or	ER+ and/or	ER+ and/or	-	-	-
PR	PR+	PR+	PR+	-	-	-
***f*HER2**	-	-	+	+	-	-
Ki-67 index	Low	High	Any	Any	Any	Any
CK 5/6	Any	Any	Any	Any	+	-

3.4. Statistical analysis

For statistical analysis the Statistical Package for the Social Sciences 20.0 for Windows (SPSS Incorporation, IBM, New York, USA) was used. A two-tailed *p* value less than 0.05 was considered statistically significant.

Analysis of variance (ANOVA) of the molecular subtypes of FMC was performed for age (in years), tumor size (in centimeters) and Ki-67 proliferation index (in percentage). When significant, differences between pairs of molecular subtypes were evaluated by the Tukey HSD method.

The Fisher's exact test was used to assess any association between the different molecular subtypes and the following clinicopathological features: age breed, reproductive status, administration of progestogens, number, location and tumor size, lymph node status, stage of disease, histopathological classification, malignancy grade, presence of necrotic areas, lymphatic invasion by neoplastic cells, lymphocytic infiltration and cutaneous ulceration. For this analysis, animals were divided in groups by age (<8 years; 8-12 years and >12 years), and tumor size (<2 cm; 2-3 cm; >3 cm), according to TNM classification (Sorenmo *et al.*, 2013). For each significant association, the odds ratio (OR) was calculated with a confidence interval (CI) of 95%.

In survival analysis, OS time was calculated, in days, from the date of the initial diagnosis to the date of death/euthanasia due to neoplasia or the date of the last follow-up for the living

queens (censored observations). Only the mammary carcinoma-related deaths were considered, while the deaths caused by other processes were censored data. The DFS time was measured, in days, from the date of the first surgery to the date of the first *in loco* regional or systemic relapse, or the death from cancer disease. Animals without relapses or lost during the follow-up were censored at the last follow-up. Animals that the moment of the relapse could not be determined by the clinician were excluded from this study. Survival curves were determined using the Kaplan-Meier method and differences between the molecular subtypes concerning survival and disease free survival time were investigated by the log-rank test.

For the animals that presented multiple mammary tumors, the lesion used in the statistical analysis was selected considering the size (the larger tumor) and the malignancy grade (the most malignant lesion), as these parameters have been already associated with poor prognosis in FMC (Weijer & Hart, 1983; Seixas *et al.*, 2011).

4. Results

4.1. Clinicopathological features

The clinical features of the 102 queens enrolled in this study are summarized in Table 14. The luminal B/HER2-negative was the most common subtype (Figure 16), with a frequency of 29.4% (30/102) followed by the luminal B/HER2-positive subtype (19.6%, (20/102). The triple negative basal-like (Figure 18) was the third most common subtype with 16.7% (17/102), closely followed by luminal A (Figure 16) with14.7% (15/102) and the triple negative normal-like subtype (Figure 18) with 12.7% (13/102). Finally, HER2-positive subtype (Figure 17) was the rarer, with a frequency of 6.9% (7/102).

The histopathological characteristics of the 229 mammary tumors, which were collected from the 102 female cats, are summarized in Table 15.

Table 14. FMC clinical and pathological characteristics and associations with the molecular subtype

Clinical features	Total (n=102)	Luminal A (n=15)	Luminal B /HER2- (n=30)	Luminal B /HER2+ (n=20)	HER2+ (n=7)	TN basal-like (n=17)	TN normal-like (n=13)	*p*
Age, years								
Mean ±SD	11.4 ±2.82	10.5 ±2.1	11.4 ±2.5	11.1 ±3.2	12.4 ±4.2	12 ±3.0	11.9±2.8	0.580
Age by group								
<8	9 (9.2%)	2 (13.3%)	1 (3.4%)	4 (20.0%)	1 (14.3%)	1 (6.2%)	0 (0.0%)	
8-12	54 (55.1%)	10 (66.7%)	20 (69.0%)	8 (40.0%)	2 (28.6%)	9 (56.2%)	5 (45.5%)	0.272
>12	35 (35.7%)	3 (20.0%)	8 (27.6%)	8 (40.0%)	4 (57.1%)	6 (37.5%)	6 (54.5%)	
Unknown*	4	0	1	0	0	1	2	
Breed								
Inbreed	82 (80.4%)	12 (80.0%)	24 (80.0%)	17 (85.0%)	7 (100%)	11 (64.7%)	11 (84.6%)	
Siamese	9 (8.8%)	1 (6.7%)	2 (6.7%)	3 (15.0%)	0 (0.0%)	3 (17.6%)	0 (0.0%)	
Persian	7 (6.8%)	1 (6.7%)	2 (6.7%)	0 (0.0%)	0 (0.0%)	2 (15.4%)	2 (15.4%)	0.834
NFC	3 (2.9%)	1 (6.7%)	1 (3.3%)	0 (0.0%)	0 (0.0%)	1 (5.9%)	0 (0.0%)	
Russian Blue	1 (1.0%)	0 (0.0%)	1 (3.3%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	
Spayed								
No	53 (53.0%)	10 (66.7%)	14 (46.7%)	8 (42.1%)	2 (28.6%)	9 (52.9%)	10 (83.3%)	
Yes	47 (47.0%)	5 (33.3%)	16 (53.3%)	11 (57.9%)	5 (71.4%)	8 (47.1%)	2 (16.7%)	0.125
Unknown*	2	0	0	1	0	0	1	
Progestogens								
No	33 (40.7%)	4 (33.3%)	12 (48.0%)	6 (42.9%)	2 (33.3%)	5 (35.7%)	4 (40.0%)	
Yes	48 (59.3%)	8 (66.7%)	13 (52.0%)	8 (57.1%)	4 (66.7%)	9 (64.3%)	6 (60.0%)	0.951
Unknown*	21	3	5	6	1	4	2	
Multiple T								
No	52 (51.0%)	9 (60.0%)	16 (53.3%)	10 (50.0%)	2 (28.6%)	8 (47.1%)	7 (53.8%)	0.833
Yes	50 (49.0%)	6 (40.0%)	14 (46.7%)	10 (50.0%)	5 (71.4%)	9 (46.2%)	6 (46.2%)	
LNS								
Negative	53 (57.0%)	9 (64.3%)	16 (61.5%)	8 (42.1%)	3 (50.0%)	9 (60.0%)	8 (61.5%)	
Positive	40 (43.0%)	5 (35.7%)	10 (38.5%)	11 (57.9%)	3 (50.0%)	6 (40.0%)	5 (38.5%)	0.773
Unknown*	9	1	4	1	1	2	0	

Stage								
I	22 (21.6%)	2 (13.3%)	6 (20.0%)	6 (30.0%)	1 (14.3%)	4 (23.5%)	3 (23.1%)	0.719
II	17 (16.7%)	5 (33.3%)	6 (20.0%)	1 (5.0%)	2 (28.6%)	2 (11.8%)	1 (7.7%)	
III	47 (46.1%)	6 (40.1%)	14 (46.7%)	10 (50.0%)	4 (57.1%)	6 (35.3%)	7 (53.8%)	
IV	16 (15.7%)	2 (13.3%)	4 (13.3%)	3 (15.0%)	0 (0.0%)	5 (29.4%)	2 (15.4%)	
Treatment								
None	9 (8.8%)	0 (0.0%)	1 (3.3%)	2 (10.0%)	0 (0.0%)	4 (23.5%)	2 (15.4%)	-
Mastectomy	86 (84.3%)	15 (100%)	26 (86.7%)	16 (80.0%)	6 (85.7%)	13 (76.5%)	10 (76.9%)	
MCh	7 (6.9%)	0 (0.0%)	3 (10.0%)	2 (10.0%)	1 (14.3%)	0 (0.0%)	1 (7.7%)	
Surgery								
Lumpectomy	12 (12.9%)	2 (13.3%)	2 (6.90%)	2 (11.1%)	3 (42.9%)	1 (7.7%)	2 (18.2%)	-
Unilateral	73 (78.5%)	12 (80.0%)	25 (86.2%)	13 (72.2%)	4 (57.1%)	10 (76.9%)	9 (81.8%)	
Bilateral	8 (8.6%)	1 (6.70%)	2 (6.90%)	3 (16.7%)	0 (0.0%)	2 (15.4%)	0 (0.0%)	
Laterality								
Left	48 (47.5%)	7 (46.7%)	12 (41.4%)	9 (45%)	4 (57.1%)	10 (58.8%)	6 (46.2%)	0.897
Right	53 (52.5%)	8 (53.3%)	17 (58.6%)	11 (55%)	3 (42.9%)	7 (41.2%)	7 (53.8%)	
Unknown*	1	0	1	0	0	0	0	

* Not considered in the calculation of the percentages neither in the statistical analysis.

Abbreviations: LNS, Lymph node status; MCh, Mastectomy plus chemotherapy; NFC, Norwegian forest cat; SD, standard deviation; T, tumor; TN, triple negative

Table 15. FMC characteristics and associations with the molecular subtype

Histopathological features	Total (n=229)	Luminal A (n=37)	Luminal B /HER2- (n=62)	Luminal B /HER2+ (n=57)	HER2+ (n=11)	TN basal-like (n=40)	TN normal-like (n=22)	*p*
Localization								0.264
M1	38 (17.0%)	2 (5.7%)	13 (21.7%)	11 (20.0%)	2 (18.2%)	6 (15.0%)	4 (18.2%)	
M2	54 (24.2%)	8 (22.8%)	18 (30.0%)	11 (20.0%)	2 (18.2%)	13 (32.5%)	2 (9.1%)	
M3	74 (32.8%)	15 (42.9%)	18 (30.0%)	16 (29.1%)	3 (27.3%)	13 (32.5%)	9 (40.9%)	
M4	57 (26.0%)	10 (28.6%)	11 (18.3%)	17 (30.9%)	4 (36.3%)	8 (20.0%)	7 (31.8%)	
Unknown*	6	2	2	2	0	0	0	
Size, cm								
Mean ±SD	2.11 ±1.59	1.59 ±1.1	1.85 ±1.29	1.84 ±1.48	2.18 ±1.6	2.86 ±2.01	3.00 ±1.74	<0.001
Size by groups								
<2	113 (49.8%)	22 (59.5%)	29 (46.8%)	34 (61.8%)	4 (36.3%)	16 (40.0%)	8 (36.4%)	
2-3	68 (30.0%)	12 (32.4%)	23 (37.1%)	12 (21.8%)	5 (45.5%)	10 (25.0%)	6 (27.3%)	0.024
>3	46 (20.2%)	3 (8.10%)	10 (16.3%)	9 (16.4%)	2 (18.2%)	14 (35.0%)	8 (36.4%)	
Unknown*	2	0	0	2	0	0	0	
HP classification								
"in situ" carcinoma	10 (4.4%)	2 (5.4%)	2 (3.2%)	5 (8.8%)	0 (0.0%)	0 (0.0%)	1 (4.5%)	0.064
TP carcinoma	135 (59.0%)	28 (75.7%)	36 (58.9%)	35 (61.4%)	4 (36.7%)	20 (50.0%)	12 (54.5%)	
Mucinous carcinoma	15 (6.6%)	0 (0.0%)	4 (6.5%)	0 (0.0%)	2 (18.2%)	4 (10.0%)	5 (22.7%)	
Cribriform carcinoma	34 (14.8%)	4 (10.8%)	9 (14.5%)	7 (12.3%)	2 (18.2%)	9 (22.5%)	3 (13.6%)	
Solid carcinoma	33 (14.4%)	2 (5.4%)	11 (17.7%)	9 (15.7%)	3 (27.3%)	7 (17.5%)	1 (4.5%)	
SC carcinoma	1 (0.4%)	0 (0.0%)	0 (0.0%)	1 (1.8%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	
Carcinosarcoma	1 (0.4%)	1 (2.7%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	
Malignancy grade								
I	28 (12.2%)	10 (27.0%)	4 (6.5%)	12 (21.1%)	0 (0.0%)	1 (2.5%)	1 (4.5%)	<0.001
II	51 (22.3%)	11 (29.7%)	15 (24.2%)	16 (28.0%)	1 (9.1%)	6 (15.0%)	2 (9.1%)	
III	150 (65.5%)	16 (43.3%)	43 (69.3%)	29 (50.9%)	10 (90.9%)	33 (82.5%)	19 (86.4%)	
Necrosis								
No	82 (35.8%)	18 (48.6%)	23 (37.0%)	28 (49.1%)	3 (27.3%)	7 (17.5%)	3 (13.6%)	0.003
Yes	147 (64.2%)	19 (51.4%)	39 (63.0%)	29 (50.9%)	8 (72.7%)	33 (82.5%)	19 (86.4%)	
Lymphatic invasion								
No	188 (82.1%)	36 (97.3%)	50 (80.6%)	44 (77.2%)	8 (72.7%)	31 (77.5%)	19 (86.4%)	0.138
Yes	41 (17.9%)	1 (2.7%)	12 (19.4%)	13 (22.8%)	3 (27.3%)	9 (22.5%)	3 (13.6%)	

LI								
No	84 (36.7%)	14 (37.8%)	25 (40.3%)	20 (35.1%)	2 (18.2%)	14 (35.0%)	9 (40.9%)	0.810
Yes	145 (63.3%)	23 (62.2%)	37 (59.7%)	37 (64.9%)	9 (81.8%)	26 (65.0%)	13 (59.1%)	
Ulceration								
No	204 (89.1%)	35 (94.6%)	57 (92.0%)	53 (93.0%)	9 (81.8%)	34 (85.0%)	16 (72.7%)	0.078
Yes	25 (10.9%)	2 (5.4%)	5 (8.0%)	4 (7.0%)	2 (18.2%)	6 (15.0%)	6 (27.3%)	
ER status								
Negative	148 (64.6%)	8 (21.6%)	39 (63.0%)	28 (49.1%)	11 (100%)	40 (100%)	22 (100%)	<0.001
Positive	81 (35.4%)	29 (78.4%)	23 (37.0%)	29 (50.9%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	
PR status								
Negative	130 (56.8%)	21 (56.8%)	15 (24.2%)	21 (36.8%)	11 (100%)	40 (100%)	22 (100%)	<0.001
Positive	99 (43.2%)	16 (43.2%)	47 (75.8%)	36 (63.2%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	
HER2 status								
Negative	161 (70.3%)	37 (100%)	62 (100%)	0 (0.0%)	0 (0.0%)	40 (100%)	22 (100%)	<0.001
Positive	68 (29.7%)	0 (0.0%)	0 (0.0%)	57 (100%)	11 (100%)	0 (0.0%)	0 (0.0%)	
Ck 5/6 status								
Negative	91 (39.9%)	14 (37.8%)	28 (45.9%)	22 (38.6%)	5 (45.5%)	0 (0.0%)	22 (100%)	<0.001
Positive	137 (60.1%)	23 (62.2%)	33 (54.1%)	35 (61.4%)	6 (54.5%)	40 (100%)	0 (0.0%)	
Unknown*	1	0	1	0	0	2		
Ki-67 index (%)								
Mean ±SD	23.5 ±17.7	6.9 ±3.9	25.4 ±10.2	20.8 ±17.7	34.5 ±13.7	31.6 ± 23.2	32.3 ± 19.1	<0.001
Ki-67 by groups								
Low	83 (36.2%)	37 (100%)	0 (0.0%)	30 (52.6%)	0 (0.0%)	11 (27.5%)	5 (22.7%)	
High	146 (63.8%)	0 (0.0%)	62 (100%)	27 (47.4%)	11 (100%)	29 (72.5%)	17 (77.3%)	<0.001

* Not considered in the calculation of the percentages neither in the statistical analysis.

Abbreviations: HP, Histopathological; SC, Squamous cell; LI, Lymphocitic infiltration; SD, standard deviation; TP, Tubulopapillary.

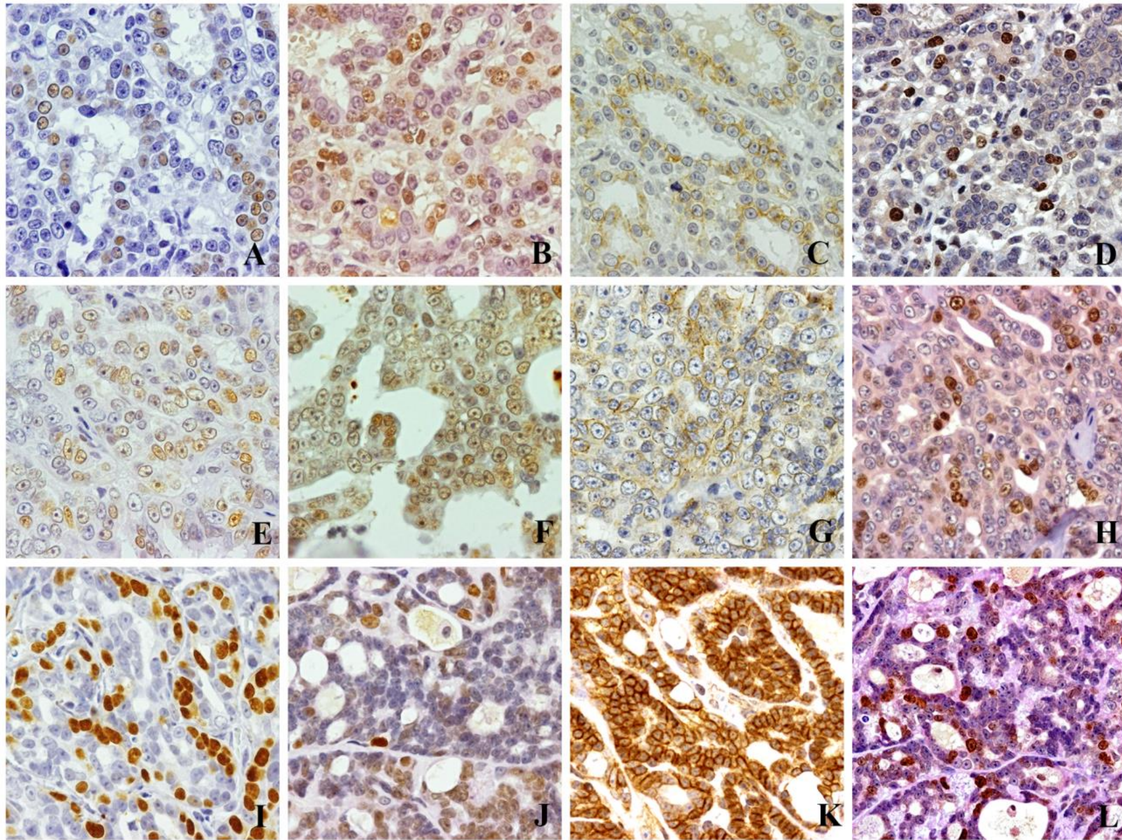

Figure 16. Immunohistochemical expression of the different proteins studied in FMC.

(A-D) Luminal A subtype in a feline tubulopapillary carcinoma with positive score for ER (A), PR (B), *f*HER2-negative status (C) and 9% Ki-67 index (D); **(E-H) Luminal B/HER2-negative subtype** in a feline tubulopapillary carcinoma presenting positive immunostaining for ER (E) and PR (F), negative score for *f*HER2 (G) and a high Ki-67 index of 21.3% (H); **(I-L) Luminal B/HER2-positive subtype** in a feline tubulopapillary carcinoma with overexpression of ER (I), PR (J), *f*HER2 (K) and with any Ki-67 index, which was estimated as 15.2% (L). Original magnification x400 (Mayer's hematoxylin).

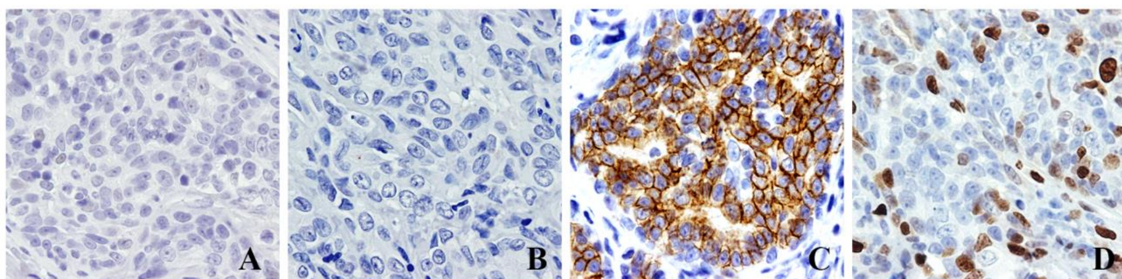

Figure 17. Immunohistochemical expression of the different proteins studied in FMC.

HER2-positive subtype in a solid mammary carcinoma. Tumor sections were negative for ER **(A)** and PR **(B)** but presented overexpression of *f*HER2 protein **(C)**. The neoplastic cells showed a high Ki-67 proliferation index of 30.8% **(D)**. Original magnification x400 (Mayer's hematoxylin).

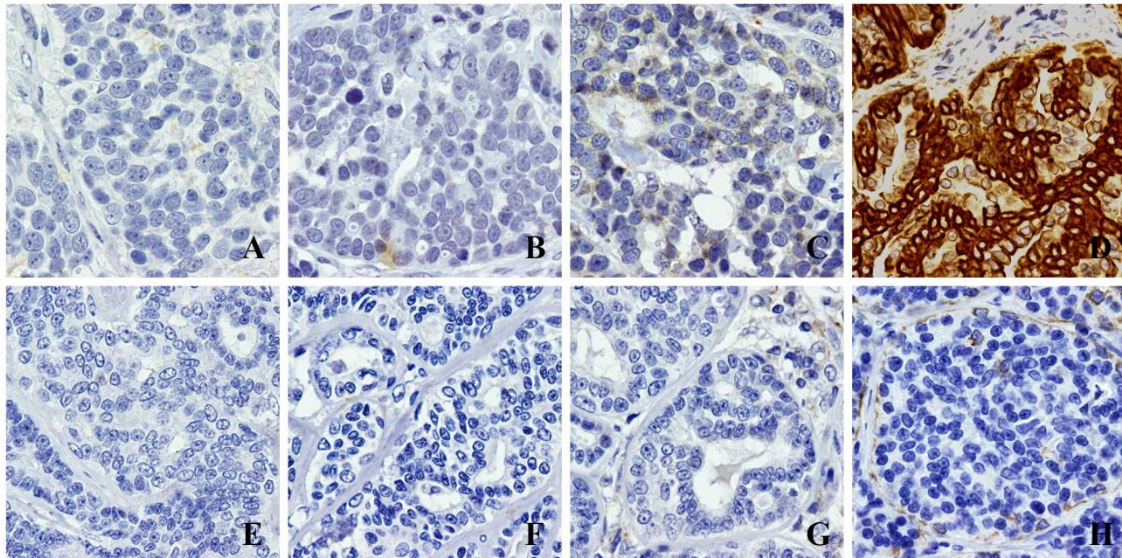

Figure 18. Immunohistochemical expression of the different proteins studied in FMC.

Triple negative subtypes in FMC. **Triple negative basal-like subtype** in a case of solid mammary carcinoma **(A-D)** being ER- **(A)**, PR- **(B)** and *f*HER2- **(C)** and positive for CK 5/6 **(D)**. **Triple negative normal-like subtype** in a case of tubulopapillary mammary carcinoma **(E-H)** being ER- **(E)**, PR- **(F)**, *f*HER2- **(G)** and CK 5/6- **(H)**. Original magnification x400 (Mayer's hematoxylin).

4.2. Associations between FMC molecular subtypes and clinicopathological features

No statistical differences were found between the molecular subtype and the clinical features (Table 14). Considering the histopathological characteristics (Table 15), tumor size (p<0.001), malignancy grade (p<0.001) and the presence of necrosis (p=0.003) revealed significant associations between the molecular subtypes.

Concerning tumor size, the triple negative basal-like and the triple negative normal-like subtypes were significantly larger when compared with other cancer subtypes (p<0.001). Furthermore, when mammary tumors were grouped according to the TNM staging system, differences between size classes were also statistical significant (p=0.024), with the luminal A subtype associated with smaller tumors (OR 3.3; 95% CI 0.95-17.6), and the triple negative basal-like subtype associated with larger tumors (OR 2.59; 95% CI 1.12-5.84).

Luminal A subtype was associated with well-differentiated tumors (OR 3.5; 95% CI 1.12-9.18) contrasting with luminal B/HER2-positive (OR 2.6; 95% CI 1.04-6.33), triple negative basal-like (OR 6.5; 95% CI 1.00-272.6) and triple negative normal-like (OR 3.6; 95% CI 1.03-19.93) subtypes that were associated with poorly differentiated tumors (grade III).

Statistical analysis also revealed that triple negative basal-like (OR 3.1, 95% CI 1.26-8.71) and triple negative normal-like subtypes (OR 3.89, 95% CI 1.09-21.18) were positively associated with tumor necrosis, contrasting with luminal B/HER2-positive tumors (OR 2.1; 95% CI 1.09-4.06) that were negatively associated.

4.3. Survival analysis

Follow-up data were available for OS study in 90 queens. The number of tumor related deaths at the end of the study was 45 (50%) and the mean overall survival was 671.7 days (SD ±63.4 days). There were no statistical differences in the mean survival time for each molecular subtype (p=0.226), but the luminal A subtype showed a better outcome, with the highest survival time (mean OS=943.6 days, SD ±166.8). It was followed by the triple negative normal-like (mean OS=725 days, SD ±163.8), luminal B/HER2-negative (mean OS=625.2 days, SD ±97.2), luminal B/HER2-positive (mean OS=568.3 days, SD ±98.2) and HER2-positive subtype (mean OS=432.3 days, SD ±108.9). Finally, the triple negative basal-like subtype presented the lowest survival time, with a OS mean of 368.9 days (SD ±83.3).

In the DFS study only 77 animals were considered. Relapse of the disease was recorded in 46 queens (59.7%, 46/77) and of these, 34 (73.9%, 34/46) presented loco regional relapse, while 12 animals (26.1%, 12/46) presented distant metastasis. The mean for disease free survival period was 464.9 days (SD ±49.9). Although it was not statistically significant (p=0.065), luminal A subtype presented the higher free disease survival time (mean 619.2 days, SD ±73.6) followed by luminal B/HER2-positive (511.1 days, SD ±113.6), luminal B/HER2-negative subtype (372.2 days, SD ±61.5), triple negative basal-like subtype (322.6 days, SD ±67), triple negative normal-like (214.4 days, SD ±42.7) and, finally, the HER2-positive subtype (203.4 days, SD ±37.8).

4.4. Concordance of the molecular subtypes of primary tumors from the same animal

In 50 queens (49%, 50/102) multiple mammary masses were present at the initial diagnosis, ranging from 2 to 5 independent tumors: 24 presented 2 mammary masses (48%, 24/50), 14 had 3 tumors (28%, 14/50), 9 showed 4 tumors (18%, 9/50) and, finally, 3 had 5 tumors (6%, 3/50).

In 18 out of the 50 cats (36%) the molecular classification was equal in the different primary tumors. The remaining 32 (64%, 32/50) presented discordant classifications between the primary masses. The most common association of phenotypes in the

same animal was the luminal B/HER2-negative and luminal B/HER2-positive that was found in 5 cases (16%, 5/32).

5. Discussion

The six molecular subtypes of mammary tumors were identified in the feline population studied with the luminal B/HER2-negative being the most common subtype (29.4%) followed by luminal B/HER2-positive (19.6%) and by triple negative basal-like subtypes (16.7%). These results differ from the published data (Wiese *et al.*, 2013), according to which the triple negative basal-like subtype was the most prevalent, but are concordant with Brunetti *et al.* (2013) that identified the luminal B subgroup as the most common in FMC.

In human breast cancer, the luminal A subtype is the most common (Vallejos *et al.*, 2010; Eroles *et al.*, 2012; Park *et al.*, 2012; Caldarella *et al.*, 2013), contrasting with HER2-positive and triple negative subtypes (basal-like and normal-like) that usually present the lowest frequencies (Park *et al.*, 2012; Caldarella *et al.*, 2013; Ribelles *et al.*, 2013).

In our study, luminal A subtype was significantly associated to a lower size and a low histological grade (Grade I), similarly to what is described in human breast cancer and in canine mammary carcinomas, in which this subtype has been associated with low histological grade, less invasive and less proliferative tumors. Also, the luminal A subtype presented the longest survival time (943.6 days) and disease free survival time (619.2 days), supporting that these tumors could be associated to a more favorable outcome in cats, like it is described for humans (Gama *et al.*, 2008; Eroles *et al.*, 2012).

Unlike others feline studies (Brunetti *et al.*, 2013; Wiese *et al.*, 2013), we have divided the luminal B type into two subtypes, luminal B/HER2-negative and luminal B/HER2-positive, as is recommended by St Gallen panel.

Despite the similar features among the two subtypes, the luminal B/HER2-positive was significantly associated to malignancy grade, presenting a 2.3 times higher odds (OR=2.3) of being a poorly differentiated tumor (Grade III), when compared to the other subtypes.

Moreover, the luminal B/HER2-positive subtype was associated with absence of necrotic areas (p=0.003, OR=2.1), contrasting with triple negative subtypes (normal and basal-like), that were significantly associated with the presence of necrosis, a characteristic usually related with more aggressive tumors, corroborating that, similarly with what is described for humans, luminal B tumors present less aggressive features, when compared with the non-luminal subtypes (Lam *et al.*, 2014).

Triple negative basal-like and normal-like subtypes were also significantly correlated to larger tumors with higher malignancy grades (Grade III), once again resembling what is described for humans. In fact, triple negative and HER2-positive subtypes are usually associated to a worse prognosis, presenting lower survival time in woman patients (Bosch *et al.*, 2010; Eroles *et al.*, 2012; Lam *et al.*, 2014). The triple negative type is generally divided in the normal-like and basal-like subtype, on basis of the expression of basal markers, like cytokeratins 5/6 (Nielsen *et al.*, 2004; Livasy *et al.*, 2006; Ribelles *et al.*, 2013; Lam *et al.*, 2014). According to St Gallen panel, approximately 80% of the triple negatives tumors have an intrinsic basal-like subtype, making the distinction between the two subtypes less significant in the human disease (Goldhirsch *et al.*, 2013). In our study, only 57% of the triple negative tumors were basal-like, supporting the maintenance of the distinction of the two subtypes, as was performed by other veterinary studies (Gama *et al.*, 2008; Sassi *et al.*, 2010; Brunetti *et al.*, 2013; Wiese *et al.*, 2013). The distinction between the two subtypes proved to be relevant, as they presented very distinct survival times in cats: the animals that presented triple negative basal-like tumors display the shorter OS (368.9 days), while the cats with normal-like tumors present the second larger OS (725 days).

The HER2-positive subtype showed the second lowest survival (432.3 days) and the lowest DFS (203.4 days) time. This subtype also demonstrated, together with the triple negative, higher proliferation capability, when compared with the luminal types (31.6% to 34.5% in the non-luminal subtypes *versus* 6.9% to 25.4% in the luminal subtypes).

One of the major difficulties in our study was the high percentage of animals with multiple mammary masses at the time of the diagnosis. Unlike humans, where synchronous bilateral breast carcinomas are uncommon, the presence of multiple masses (usually in the same mammary chain) at the moment of the diagnosis is very common in cats, with previous studies pointing to a prevalence higher than half of the cases (Saad *et al.*, 2008; Sorenmo *et al.*, 2013). This represents a serious difficulty, either for the interpretation of the results and comprehension of tumor disease behavior, as for disease staging, clinical and therapeutic management.

One of the objectives of our study was to evaluate if the different primary mammary lesions in the same female cat presented the same molecular phenotype. The implications of the high heterogeneity of the mammary tumors require more studies, in order to improve the management of the disease and to increase the basic knowledge of cancer behavior.

6. Conclusion

In this study, we demonstrated the molecular heterogeneity of feline mammary carcinomas, and we were able to identify and characterize 6 different molecular subtypes, presenting several similarities with the human breast cancer disease. In fact, clinicopathological characterisation of the different subtypes was similar to what is described for human medicine, what lead us to believe that FMC would be a suitable natural model for the study of human breast cancer disease, especially for luminal B/HER2-negative, the luminal B/HER2-positive and for the triple negative basal-like subtypes.

For veterinary clinical practice, this classification open perspectives for better prognostic evaluation and towards the development of new and more specific treatments, directed against target proteins, which will improve the overall and the disease free survival of the feline patients with mammary cancer.

Chapter IV – St Gallen molecular subtypes in feline mammary carcinoma and paired metastases-disease progression and clinical imlications from a 3-year follow-up study

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1. Abstract

Considering that scarce data are available on disease progression of feline mammary carcinoma (FMC), this study aimed to analyze the clinical, pathological, and immunophenotypic features collected from 61 queens with FMC and to compare the concordance ratios of the expression levels of five molecular markers (ER, PR, *f*HER2, CK5/6, and Ki-67) between primary tumors (PT) and metastatic lesions. The results showed that cats with luminal A mammary carcinomas (MC) had higher overall survival (924.6 days, p=0.001) and longer disease-free period (385.4 days, p=0.005) compared to the ones with other MC subtypes. In fact, queens with triple negative/basal-like MC showed the lowest survival (mean 156.2 days) and the shortest disease-free survival (mean 28 days) among the molecular subtypes of MC. The lung was the organ most frequently affected by metastases, and animals with lung and/or pleural metastases were more likely to display metastases at three or more locations (p=0.039). A large heterogeneity in protein expression levels was found between PT and paired metastases, with both estrogen and progesterone receptors more likely to be downregulated in metastases. Paired metastases frequently had higher Ki-67 index than PT, whereas *f*HER2 overexpression was seen in 46 samples (30%) and CK5/6 expression was found in 50.7% of metastases (36/71). Results also revealed that disease progression leads to a high percentage of triple negative/basal-like metastases (9/23; 39.1%) associated with the absence of luminal A subtype in distant metastases (0/23). This study highlights the prognostic importance of immunophenotyping of MC in cats, although the modified protein expression identified in metastases contributes to justify why possible targeted therapies may fail in some animals with metastatic disease. Altogether, the results obtained also demonstrate that FMC can be used as a model to study human breast cancer.

Keywords: Feline mammary carcinoma; Metastatic disease; Molecular classification; Prognostic biomarkers

2. Introduction

Feline mammary tumors (FMT) are the third most common tumor in cats, representing approximately 17% of all neoplasms in this species (Sorenmo *et al.*, 2013). Feline mammary carcinomas (FMC) are by far the most representative lesion within this group of tumors, showing a high metastatic potential (Sorenmo *et al.*, 2013; Zappulli *et al.*, 2015). Up to now, very limited data are available regarding disease progression in FMC, although metastatic lesions are known to be the main cause of death, representing a major clinical and therapeutic challenge (Weigelt *et al.*, 2003; Montel *et al.*, 2005; Suzuki & Tarin, 2007).

In breast cancer patients, recent studies have shown contradictory results, with some authors reporting a similar gene expression profile between the primary tumor and the paired metastases (Weigelt *et al.*, 2003; Chan *et al.*, 2012), while the majority demonstrates a marked heterogeneity of the molecular signatures between primary and metastatic lesions (Montel *et al.*, 2006; Aitken *et al.*, 2010; Aurilio *et al.*, 2014; Raica *et al.*, 2014). These data are relevant to optimise patients' management since prescribed targeted therapies consider the breast tumor subtype (Niikura *et al.*, 2012; Falck *et al.*, 2013; Yao *et al.*, 2014).

In cats, recent studies showed that several biomarkers have prognostic value in mammary carcinoma (de las Mulas *et al.*, 2000; Millanta *et al.*, 2005a; Millanta *et al.*, 2006a; Rasotto *et al.*, 2011; Seixas *et al.*, 2011; Peñafield-Verdu *et al.*, 2012; Soares *et al.*, 2013a; Maniscalco *et al.*, 2012), although the immunophenotyping of mammary tumors failed to add significant prognostic value (Brunetti *et al.*, 2013; Soares *et al.*, 2013b; Beha *et al.*, 2014).

In this study, clinical information collected from a study population of 61 queens with mammary carcinoma was evaluated, and the gene expression profiles of primary mammary carcinomas were compared with the ones obtained in local, regional, and distant metastases. Finally, correlation studies were performed between the clinicopathological features and the expression of biomarkers and also between the clinicopathological features and the molecular subtypes of the primary tumor/metastases.

3. Material and methods

3.1. Animal patients

Owners gave permission to collect samples from their pets and to use the animal's clinical data. Tumor samples were collected in accordance with the EU Directive 2010/63/EU and research was approved by the ethics committee of the Faculty of Veterinary Medicine (FVM), University of Lisbon (ULisboa).

The cohort study enrolled 61 queens with spontaneous mammary carcinomas, presented at the Teaching Hospital of the FVM-ULisboa from June 2011 to May 2014. Only cats that had one form of disease (local relapse, regional metastases, or distant metastases), at the time of the diagnosis or during the follow-up period, were included in the study. During the follow-up, animals were examined at least once every 6 months. Although 126 cats were initially selected for this study, 65 were excluded because nine showed benign mammary tumors, 31 with mammary carcinomas lacked any form of disease progression, 21 had failed the clinical periodic examinations, and four animals died from other diseases.

In order to maximize survival, a complete surgical resection of the primary tumor with tumor-free margins of 2 cm was performed in all cats that underwent to surgery (51/61, 83.6%). The remaining ten cats did not receive any treatment once they already presented distant metastases. From the selected animals (61/126), 21 (34.4%) had local relapse during the follow-up period, and 48 (78.7%) showed lymph node involvement at the time of the first diagnosis. A complete necropsy was performed in 23 (37.7%) animals in the Anatomical Pathology Laboratory (FMV-ULisboa). Tissue samples from the primary tumor and, whenever possible, from metastases (regional lymph nodes and distant organs) were fixed in 10% buffered formalin for 24 to 48 h and embedded in paraffin blocks. The following clinical data were recorded when available: age, breed, reproductive status (intact vs. spayed), administration of progestogens, number and location of tumors, tumor stage according to TNM system (Sorenmo *et al.*, 2013), prescribed treatment (none, mastectomy, mastectomy combined with chemotherapy), overall survival (OS), and disease-free survival (DFS) times.

3.2. Histopathological and immunohistochemical analysis

All tumor lesions were classified according to the World Health Organization classification system, and malignancy grade was determined by the use of two different systems: the Elston and Ellis scoring system and a novel grading system for evaluation of invasive FMC published by Mills *et al.* (2015), which considers lymphovascular invasion, nuclear form, and mitotic count (Elston & Ellis, 1998; Misdorp *et al.*, 1999;

Mills *et al.*, 2015). The presence of necrotic areas, lymphatic vessel invasion by tumor cells, lymphocytic infiltration, and skin ulceration were also evaluated.

All primary tumors and paired metastases (local, regional, and/or distant) were submitted to immunohistochemistry (IHC) using the Novocastra Novolink Polymer Detection System (Leica Biosystems) to detect estrogen (ER) and progesterone receptors (PR), feline homologue of human epidermal growth factor receptor type 2 (*f*HER2), cytokeratin 5/6 (CK5/6) and Ki-67, as summarized in Table 16 and as previously described (de las Mulas *et al.*, 2000; Brunetti *et al.*, 2013; Soares *et al.*, 2013b).

Table 16. Primary antibodies and immunohistochemical protocols used for ER, PR, *f*HER2, CK5/6 and Ki-67 detection.

Primary antibody	Dilution	Incubation	Antigen retrieval method
Mouse anti-ER-α, clone 6F11[a]	1:100	O/N, 4ºC	Pressure cooker (2 atm, 2’) with sodium citrate buffer
Rabbit anti-PR, clone 1E2[b]	RTU	O/N, 4ºC	Water bath (95ºC, 60’) with sodium citrate buffer
Mouse anti-HER2, clone CB11[c]	1:200	O/N, 4ºC	Pressure cooker (2 atm, 2’) with sodium citrate buffer
Mouse anti-CK5/6, clone D5/16 B4[a]	RTU	O/N, 4ºC	Microwave (900W, 15’) with Tris-EDTA buffer (pH 9.0)
Rabbit anti-Ki-67, polyclonal[a]	1:500	60 min, RT	Pressure cooker (2 atm, 2’) with sodium citrate buffer

Abbreviations: O/N, overnight; RT, room temperature; RTU, ready-to-use.
[a] Thermo Scientific, Rockford, USA
[b] Ventana, Tucson, USA
[c] Invitrogen, Carlsbad, USA.

Briefly, the anti-ER antibody (clone 6 F11, 1:100 dilution; Thermo Scientific, Rockford, USA), the anti-HER2 antibody (cloneCB11, 1:200 dilution; Invitrogen, Carlsbad, USA),and the anti-Ki-67 polyclonal antibody (1:500 dilution; Thermo Scientific) were applied to the samples after antigen retrieval in sodium citrate buffer (0.01 M NaCH3COO, pH 6.0), in a pressure cooker for 2 min at 2 atm. For ER and fHER2 detection, the antibodies were incubated overnight at 4°C, whereas a 60-min incubation period, at room temperature, was performed for Ki-67. PR staining was conducted with the ready-to-use 1E2 antibody (Ventana, Tucson, USA), incubated overnight at 4°C, after antigen retrieval in 0.01 M sodium citrate solution (pH 6.0) in a water bath at 95°C, for 60 min. The anti-CK 5/6 antibody (clone D5/16 B4, ready to

use; Ventana) was applied overnight, at 4 °C after a heat-induced antigen retrieval procedure (microwave for 15min) with Tris-EDTA buffer (pH 9.0).

Biomarker expression from IHC assays was scored by two independent observers who were blinded to the clinicopathological features of the samples and to prognostic outcome of the queens. Discordant interpretations were further debated and settled using a multiviewer microscope.

Tissue samples were considered positive for ER or PR when the Allred score was equal or higher than 3 (Harvey *et al.*, 1999; Mohsin *et al.*, 2004; Soares *et al.*, 2013b). Quantitative immunohistochemical evaluation of *f*HER2 expression was performed by using the HercepTest scoring system (Wolff *et al.*, 2013) and samples with a score of 2 or 3 were considered positive (Millanta *et al.*, 2005a; De Maria *et al.*, 2005; Órdas *et al.*, 2007; Rasotto *et al.*, 2011; Santos *et al.*, 2013; Soares *et al.*, 2013a). Tissue samples were considered to be positive for CK5/6 whenever more than 1% of the tumor cells showed positive staining in the cytosol and/or in the cytoplasmic membrane (Brunetti *et al.*, 2013). The Ki-67 index was estimated by dividing the number of tumor cells with positive nuclear staining by the total number of tumor cells analyzed (at least 1000 cells), and samples were considered highly proliferative when at least 14% of the tumor cells stained positive for Ki-67 (Soares *et al.*, 2016b).

All microscope procedures were performed using an Olympus BX51 light microscope (Pennsylvania, USA) equipped with an Olympus DP25 camera for image capturing. The images were analyzed using Image J (Open Source Software, version 1.46r, National Institutes of Health, Bethesda, USA).

3.3. Definition of the molecular subgroups

Feline mammary carcinomas were grouped in six immunophenotypes using the following recommendations of the St Gallen International Expert Consensus panel (Goldhirsch *et al.*, 2013): luminal A (ER+ and/or PR+, *f*HER2−, any CK5/6 status, and low Ki-67 index), luminal B/HER2-negative (ER+ and/or PR+, *f*HER2−, any CK5/6 status, and high Ki-67 index); luminal B/HER2-positive (ER+ and/or PR+, *f*HER2+, any CK5/6 status, and any Ki-67 index); HER2-positive (ER−, PR−, *f*HER2+, any CK5/6 status, and any Ki-67 index); triple negative/basal-like (ER−, PR −, *f*HER2−, CK5/6+, and any Ki-67 index) and triple negative normal-like (ER−, PR −, *f*HER2−, CK5/6-, and any Ki-67 index).

3.4. Statistical analysis

Statistical analysis was performed using SPSS 20.0 for Windows (SPSS Incorporation, IBM) and p values less than 0.05 ($p<0.05$) were considered to be statistically significant.

To analyze continuous variables, cases were divided in the following groups: <10 years and ≥10 years; tumors of <2 cm; 2 up to 3 cm; >3 cm; tumors with a Ki-67 index ≥14% or of less than 14%. Local relapse (LR) was defined as local relapse in the homolateral mammary chain or in the ipsilateral glands. Regional metastases (RM) were defined as presence of tumor cells in the regional lymph nodes (axillary or inguinal), and distant metastases (DM) defined whenever tumor tissue was present in any organ apart from the regional lymph nodes and the mammary glands.

OS time was calculated, in days, from the date of the initial diagnosis to the date of death/euthanasia due to neoplasia or the date of the last follow-up for the living queens (censored observations). DFS time was also estimated in days, from the date of the first surgery to the date of diagnosis of LR/RM/DM, or to the date of death/euthanasia due to cancer disease progression. Survival analysis was performed only with the animals that underwent mastectomy, while animals without any treatment or submitted to chemotherapy were excluded from this analysis. Additionally, cats that died from other diseases and animals without relapse or lost during the follow-up were also censored. Survival analysis was performed using Kaplan-Meier curves, and differences in the OS and the DFS were investigated by the log-rank test. Multivariate analysis using the Cox proportional hazard model was performed to determine the way in which OS and DFS were affected by the other co-variables. The hazard ratio (HR) was calculated with a 95% confidence interval (95% CI).

Associations between the different clinicopathological features were analyzed using the Fisher's exact test. Finally, the concordance of ER/PR/*f*HER2/CK5/6 status and Ki-67 index was compared between the primary tumor (PT) and the LR, between the PT and the RM, between the PT and the DM. In order to ensure the consistency of our results, the statistical analysis of the relapse lesions, regional and distant metastases, as well as the concordance determination were performed on cats that underwent mastectomy only.

4. Results

4.1. Animal population

A total of 61 queens with a mean age of 11.8 ± 0.34 years were enrolled in this prospective study, and their clinicopathological features are summarized in Tables 17 and 18. Among these animals, 21 (34.4%) showed a local relapse, 48 (78.7%) lymphnode metastases, and 23 (37.7%) had distant metastases. Fifty-one animals (83.6%) were submitted to mastectomy, whereas the remaining ten (16.4%) did not receive this procedure. Additionally, eight were treated with anthracycline-based chemotherapy after mastectomy (1 mg/kg of doxorubicin, intravenously, every 3 weeks for a maximum of five treatments or until the cat developed progressive disease or concurrent illness).

At the moment of the first diagnosis, most of the mammary carcinomas were classified as stage III (62.3%, 38/61) or IV (23%, 14/61), and some as stage I (8.2%, 5/61) or stage II (6.6%, 4/61).The mean PT size was 2.9 cm ± 0.34, whereas LR lesions had a mean size of 1.86 cm (SD=0.34 cm). Primary tumors and LR lesions were mostly classified as tubulopapillary carcinomas (65.6 and 42.9%, respectively), and were poorly differentiated (88.5 and 61.9%, respectively). According to the Elston & Ellis classification system (Elston & Ellis, 1998), most of the primary tumors were poorly differentiated (88.5%, 54/61), with only a small percentage being moderately differentiated (9.8%, 6/61) or well differentiated (1.6%, 1/61). Using the novel grading system for evaluation of invasive FMC (Mills *et al.*, 2015), the results obtained were quite similar, with most of the primary tumors showing high grade malignancy (57.4%, 35/61), followed by intermediate grade (34.4%, 21/61) and low grade MC (8.2%, 5/61). Concerning local relapses, we found a decrease in the percentage of grade III carcinomas (61.9%,13/21, using the Elston & Ellis classification, and 42.8%, 9/21, using the novel grading system) and an increase of grade I carcinomas (14.3%, 3/61 or 28.6%, 6/21, using the Elston & Ellis or the novel classification, respectively).

Table 17. Clinical features of the cats diagnosed with mammary carcinoma (n=61).

Features	Number of animals (%)
Age, mean ± SD (years)	11.8 ± 0.34
<10 years	12 (20.7%)
≥10 years	46 (79.3%)
Unknown[1]	3
Breed	
Undetermined	44 (80.3%)
Siamese	5 (8.2%)
Norwegian Forest Cat	4 (6.6%)
Persian	2 (3.3%)
Blue Russian	1 (1.6%)
Spayed	
No	27 (45.8%)
Yes	32 (54.2%)
Unknown[1]	2
Progestogens	
No	19 (44.2%)
Yes	24 (55.8%)
Unknown[1]	18
Treatment	
None	10 (16.4%)
Mastectomy	43 (70.5%)
Mastectomy plus chemotherapy	8 (13.1%)
Multiple mammary tumors	
No	28 (45.9%)
Yes	33 (54.1%)
Lymph node status	
Negative	12 (21.1%)
Positive	45 (78.9%)
Unknown[1]	4
Disease stage (TMN)	
I	5 (8.2%)
II	4 (6.6%)
III	38 (62.3%)
IV	14 (23.0%)
Tumor location	
M1	9 (15.0%)
M2	10 (16.7%)
M3	21 (35.0%)
M4	20 (33.3%)
Unknown[1]	1

[1] Not considered in the calculation of the percentages.

Table 18. Tumor features found in the studied cats.

Features	Number of animals (%)			
	PT[8] (n=61)	LR[9] (n=21)	RM[10] (n=48)	DM[11] (n=23)
Tumor size, mean ± SD[1] (cm)	2.90 ± 0.21	1.86 ± 0.34	-	-
<2 cm	14 (23.0%)	12 (57.1%)		
2-3 cm	24 (39.3%)	4 (19.0%)		
>3 cm	23 (37.7%)	5 (23.9%)		
HP[2] classification			-	-
Tubulopapillary carcinoma	40 (65.6%)	9 (42.9%)		
Solid carcinoma	8 (13.1%)	3 (14.3%)		
Cribriform carcinoma	8 (13.1%)	5 (23.8%)		
Mucinous carcinoma	5 (8.2%)	0 (0.0%)		
“in situ” carcinoma	0 (0.0%)	3 (14.3%)		
Squamous cell carcinoma	0 (0.0%)	1 (4.8%)		
MG[3] (EE[4] system)			-	-
I	1 (1.6%)	3 (14.3%)		
II	6 (9.8%)	5 (23.8%)		
III	54 (88.5%)	13 (61.9%)		
MG[3] (Mills *et al.* system)			-	-
I	5 (8.2%)	6 (28.6%)		
II	21 (34.4%)	6 (28.6%)		
III	35 (57.4%)	9 (42.8%)		
Necrosis			-	-
No	14 (23.0%)	7 (33.3%)		
Yes	47 (77.0%)	14 (66.7%)		
Lymphatic invasion			-	-
No	45 (73.8%)	17 (81.0%)		
Yes	16 (26.2%)	4 (19.0%)		
Lymphocytic infiltration			-	-
No	11 (18.0%)	10 (47.6%)		
Yes	50 (82.0%)	11 (52.4%)		
Ulceration			-	-
No	51 (83.6%)	21(100.0%)		
Yes	10 (16.4%)	0 (0.0%)		
ER status				
Negative	48 (78.7%)	11 (52.4%)	42 (87.5%)	22 (95.7%)
Positive	13 (21.3%)	10 (47.6%)	6 (12.5%)	1 (4.3%)
PR status				
Negative	29 (47.5%)	10 (47.6%)	24 (50.0%)	16 (69.6%)
Positive	32 (52.5%)	11 (52.4%)	24 (50.0%)	7 (30.4%)
***f*HER2 status**				
Negative	42 (68.9%)	12 (57.1%)	36 (75.0%)	17 (73.9%)
Positive	19 (31.1%)	9 (42.9%)	12 (25.0%)	6 (26.1%)
CK5/6 status				
Negative	31 (51.7%)	11 (52.4%)	22 (45.8%)	13 (56.5%)
Positive	29 (48.3%)	10 (47.6%)	26 (54.2%)	10 (43.5%)
Ki-67 index, mean ±SD[1] (%)	26.7 ± 2.2	22.9 ± 2.9	32.2 ± 2.6	36.0 ± 3.9
Low	14 (23.0%)	6 (28.6%)	8 (16.7%)	2 (8.7%)
High	47 (77.0%)	15 (71.4%)	40 (83.3%)	21 (91.3%)
Molecular Classification				
Luminal A	8 (13.1%)	2 (9.5%)	4 (8.3%)	0 (0.0%)
Luminal B/HER2-[5]	18 (29.5%)	8 (38.1%)	14 (29.2%)	2 (8.7%)
Luminal B/HER2+[6]	14 (23.0%)	7 (33.3%)	10 (20.8%)	6 (26.1%)
HER2-positive	4 (6.6%)	2 (9.5%)	3 (6.3%)	0 (0.0%)
TN[7] basal-like	10 (16.4%)	1 (4.8%)	11 (22.9%)	9 (39.1%)
TN[7] normal-like	7 (11.5%)	1 (4.8%)	6 (12.5%)	6 (26.1%)

[1]SD (standard deviation); [2]HP (histopathological); [3]MG (malignancy grade); [4]EE (Elston & Ellis); [5]Luminal/HER2- (Luminal B/HER2 negative); [6]Luminal/HER2+ (Luminal B/HER2 positive); [7]TN

(Triple negative); [8]PT (Primary Tumor); [9]LR (Local relapse); [10]RM (Regional Metastases); [11]DM (Distant Metastases)

4.2. Distribution pattern of subtypes and metastases

Both luminal B/HER2-negative (Figure 19, a2–e2) and luminal B/HER2-positive (Figure 19, a3–e3) were the most frequent subtypes in primary and relapse mammary carcinomas, although the triple negative/basal-like subtype (Figure 19, a5–e5) was the most common in DM (Table 18).

Ninety-three distant metastases were identified in 13 different locations (Table 19) and, interestingly, cats with lung and pleural metastases were more prone to having metastatic lesions in three or more sites (p=0.039).The lung and/or pleura (87%), non-regional lymph nodes (70%), and liver (30.4%) were the most affected organs. Of the cats with metastatic disease, 34.8% (8/23) had one or two metastatic sites, while 43.5% (10/23) had three to five metastatic sites, and 21.7% (5/23) had six to eight sites.

No association was found between the features of the primary tumor (histological type, malignancy grade, presence of necrotic areas, presence of lymphatic invasion by neoplastic cells, presence of lymphocytic infiltration, skin ulceration, biomarker status, and molecular classification) and the presence of lung metastases or the number of metastases per animal.

Table 19. Location and number of metastases found at necropsy of 23 cats with mammary carcinoma.

Metastases location	Number of animals (%)
Lung and/or pleura	20 (87.0%)
Non-regional lymph nodes	16 (70.0%)
Liver	7 (30.4%)
Skin	5 (21.7%)
Urinary system	4 (17.4%)
Cardiac muscle and/or pericardium	3 (13.0%)
Muscle	3 (13.0%)
Pancreas	3 (13.0%)
Spleen	2 (8.7%)
Adrenal glands	2 (8.7%)
Thyroid gland	2 (8.7%)
Gynae organs	2 (8.7%)
CNS	1 (4.3%)
Number of metastases per animal	**Number of animals (%)**
0-2	8 (34.8%)
3-5	10 (43.5%)
6-8	5 (21.7%)

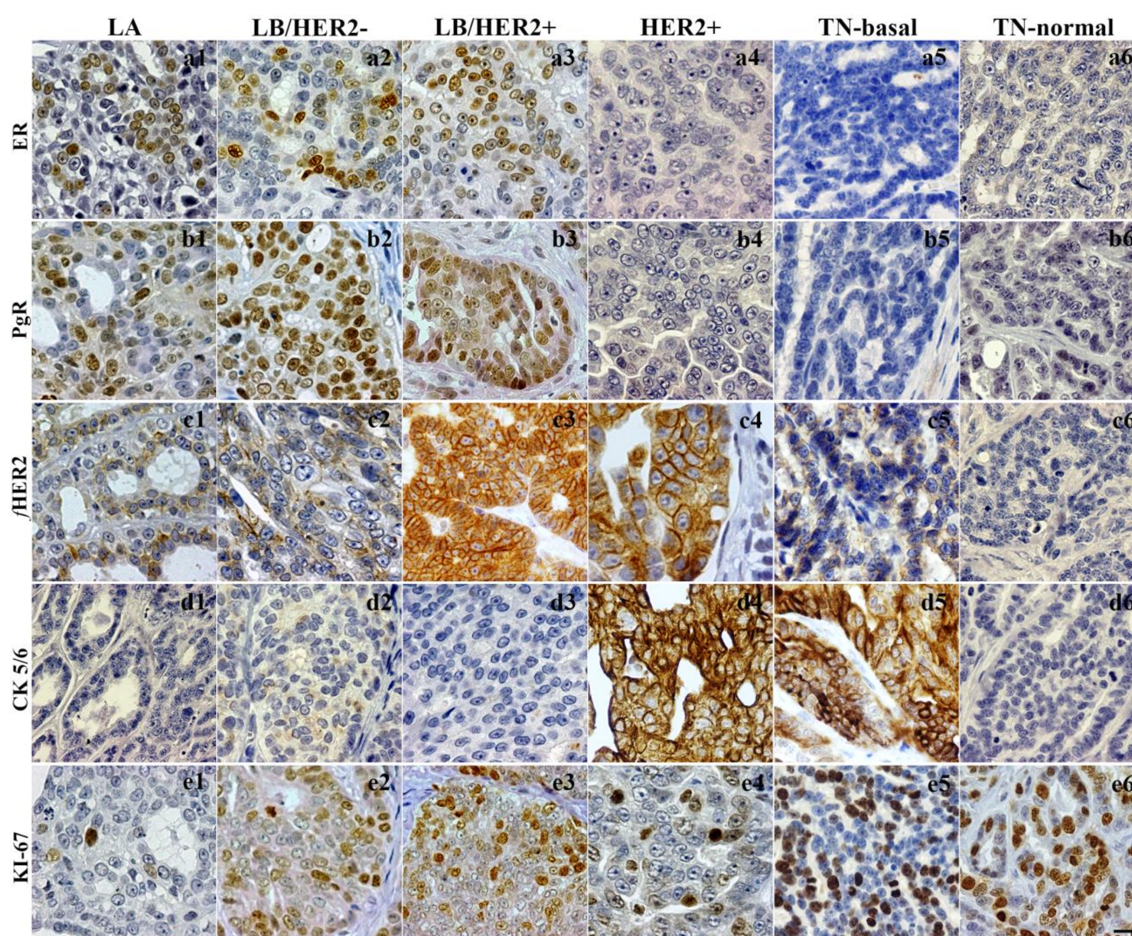

Figure 19. Immunohistochemical classification of FMC into six subtypes on the basis of their ER, PR, *f*HER2, CK5/6, and Ki-67 expression.

Luminal A FMC were defined as ER+ **(a1)** and/or PR+ **(b1)**, *f*HER2- **(c1)**, CK5/6 **(d1)** and a Ki-67 index of <14% **(e1)**. Luminal B/HER2-negative FMC were ER+ **(a2)** and/or PR+ **(b2)**, *f*HER2- **(c2)**, CK5/6- **(d2)** and a Ki-67 index of 14% or greater **(e2)**. FMC that were ER+ **(a3)** and/or PR+ **(b3)**, *f*HER2+ **(c3)**, CK5/6- **(d3)** and showed a Ki-67 index of ≥14% **(e3)** were classified as luminalB/HER2-positive tumors. HER2-positive mammary carcinomas were ER- **(a4)**, PR- **(b4)**, *f*HER2+ **(c4)** and CK5/6+ **(d4)**, showing a Ki-67 index <14% **(e4)**. Triple negative/basal-like mammary carcinomas were ER- **(a5)**, PR- **(b5)**, *f*HER2- **(c5)**, CK5/6+ **(d5)** and a Ki-67 index of ≥14% **(e5)**. Triple negative normal-like mammary carcinomas showed low expression of ER **(a6)**, PR **(b6)**, *f*HER2 **(c6)**, and CK5/6 **(d6)** and a Ki-67 index equal or higher than 14% **(e6)**. *Scale bar*=20μM

4.3. Survival analysis

Follow-up data concerning OS time was available for only 59 cats (559.7 ± 65.1 days), since two had died from other diseases and were not considered for the survival analysis.

During the follow-up period, 40 animals were euthanized due to disease progression and despite the prescribed therapy (392 ± 83.2 days). When the animals were divided

according to the treatment prescribed, a significant difference in OS was found ($p<0.0001$) when comparing the animals without treatment (mean OS 179.9 ± 82.5 days) versus those submitted only to mastectomy (559.9 ± 76.1 days) or those submitted to surgery and chemotherapy (949.5 ± 143.9 days).

Cats diagnosed with triple negative/basal-like or HER2-positive MC showed a significantly shorter survival time, when compared to other subtypes (156.2 ± 70.9 days; 200.3 ± 93.1 days, respectively; p=0.001) (Figure 20a). Furthermore, animals with luminal A carcinomas showed the best survival (924.6 ± 200.9 days), followed by queens with triple negative normal-like MC (725.3 ± 276.5 days), luminal B/HER2-negative mammary carcinomas (448.1 ± 71.5 days), and animals with luminal B/HER2-positive mammary tumors (447.1 ± 61.4 days). In addition, cats with negative ER and/or positive CK5/6 status and/or high Ki-67 index (≥14%) were significantly associated with lower OS (p=0.04, p=0.022, p=0.011, respectively; supplementary Table 1).

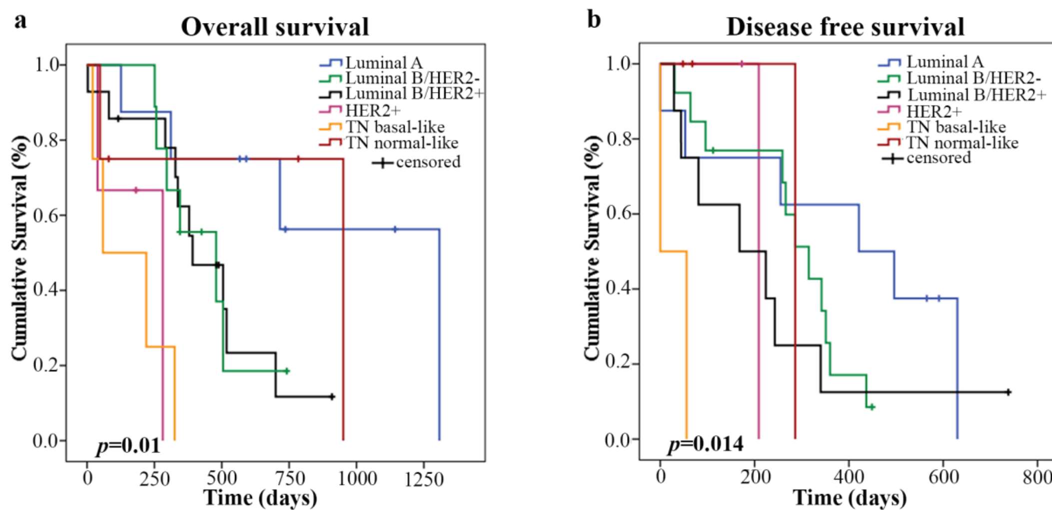

Figure 20. Kaplan-Meier curves illustrating the OS (a) and DFS (b) of cats subjected to mastectomy (without chemotherapy) stratified according to the mammary tumor subtype.

Cats with luminal A mammary carcinomas showed the longer OS (median 1308 days) and DFS period (median 421 days). In contrast, cats with triple negative/basal-like and HER2-positive mammary carcinomas experienced the shortest survival times (median 59 and 281 days, respectively), and the shortest DFS period (median 28 and 209 days, respectively). Cats with luminal B/HER2-positive mammary carcinomas showed intermediate survival times (median of 479 days), being followed by cats with luminal B/HER2-negative mammary carcinomas (median of 392 days) and by cats with triple negative/normal-like tumors (median of 951 days). Cats with luminal B/HER2-negative and triple negative normal-like mammary carcinomas showed the second longest DFS (315 and 286 days, respectively), followed by cats with HER2-positive carcinomas (median of 168 days)

The DFS ranged from 222.3 to 284.9 days with a mean value of 253.6 days (n=48) and a median of 243 ± 30 days. For the DFS study only the group of animals subjected to surgery (without chemotherapy) was considered, in order to ensure sample homogeneity. The univariate analysis showed that cats with triple negative/basal-like MC had the shortest DFS (28 ± 28 days, p=0.014, Figure 20b), followed by the group of cats with HER2-positive mammary carcinomas (209 ± 25.5 days), the group with luminal B/HER2-positive tumors (233.4 ± 76.2 days), the luminal B/HER2-negative group (276.6 ± 36.5 days), the triple negative normal-like group (286 ± 132 days) and, finally, by the group of cats with luminal A tumors (389.4 ± 93.8 days).

4.4. Multivariable analysis

All variables that showed association in the univariate survival analysis (p<0.05) were included in the multivariable analysis (breed, number of primary tumors, stage, tumor size, malignancy grade using the Mills' score system, necrosis, lymphatic invasion, lymphocytic invasion, skin ulceration, ER status, CK 5/6 status, and Ki-67 index levels). Cox regression confirmed that molecular classification is an independent indicator of clinical outcome (p=0.009, Table 20) for OS but not for DFS (p=0.398, Table 21).
This multivariate analysis confirmed that a queen presenting a HER2-positive or a triple negative/basal-like mammary carcinoma has a higher risk of dying when compared to the other molecular subtypes (HR 438.4 [95% CI, 14–13681.7] and HR 210.9 [95% CI, 4.8-9352.3], respectively). In addition, the multivariate cox regression hazard analysis also showed that Ki-67 index levels (p=0.035) and the tumor size (p=0.001) were independent prognostic factors related to OS, with animals that had tumors with high Ki-67 index values or large size having a greater risk of dying from cancer disease (HR=34.6 [95% CI, 1.3-926.5] and HR=3.1 [95% CI, 1–9.3], respectively). Considering the DFS multivariate analysis, only the presence of skin ulceration (HR=7.8 [95% CI, 1.2-52.4], p=0.034) has proven to be an independent predictor. No other variables independently affected OS or DFS in the multivariate survival study.

Table 20. Multivariate cox regression analysis for OS in cats that underwent only to surgery (n=42).

	Overall survival				
	n	HR[5]	95% CI[6] Lower	Upper	*p*
Breed	42	0.13	-	-	0.135
Disease stage (TMN)	42	4.1	1	17.1	0.05
Tumor size					
<2 cm	10	0.023	0.002	0.2	0.001*
2-3 cm	19	3.1	1	9.3	0.048*
>3 cm	13	*Reference*			
MG[1] (Mills *et al.* system)	42	0.2	0.01	1.9	0.148
Lymphatic invasion	42	3.9	0.7	21.2	0.114
ER status	42	2.1	0.3	15.9	0.482
CK5/6 status	42	0.8	0.2	3.5	0.76
Ki-67 index					
Low	14	*Reference*			
High	28	34.6	1.3	926.5	0.035*
Molecular Classification					0.009*
Luminal A	8	3.5	0.2	106.4	0.467
Luminal B/HER2-[2]	14	55.9	3.5	886.5	0.004*
Luminal B/HER2+[3]	9	55.7	2.1	1467.8	0.016*
HER2-positive	3	438.4	14	13681.7	0.001*
TN[4] basal-like	4	210.9	4.8	9352.3	0.006*
TN[4] normal-like	4	*Reference*			

[1]MG (malignancy grade); [2]Luminal/HER2- (Luminal B/HER2 negative); [3]Luminal/HER2+ (Luminal B/HER2 positive); [4]TN (Triple negative); [5]HR (Hazard ratio); [6]CI (confidence interval)

Table 21. Multivariate cox regression analysis for DFS in cats that underwent only to surgery (n=36).

	Disease-free survival				
	n	HR[5]	95% CI[6] Lower	Upper	*p*
Disease stage (TMN)	36	1.2	0.5	3.1	0.686
Tumor size	36	1.3	0.5	3.3	0.608
MG[1] (Mills *et al.* system)	36	1.5	0.4	5.6	0.581
Necrosis	36	3.2	0.7	14.2	0.118
Lymphocytic invasion	36	4.9	0.9	27.6	0.078
Ulceration					
No	30	*Reference*			
Yes	6	7.8	1.2	52.4	0.034*
Molecular Classification					0.398
Luminal A	8	.32	0.009	10.9	0.528
Luminal B/HER2-[2]	13	1.4	0.1	18.4	0.787
Luminal B/HER2+[3]	8	1.1	0.1	13.4	0.918
HER2-positive	2	2.1	0.2	22.6	0.531
TN[4] basal-like	3	10.5	0.3	326.2	0.181
TN[4] normal-like	2	*Reference*			

[1]MG (malignancy grade); [2]Luminal/HER2- (Luminal B/HER2 negative); [3]Luminal/HER2+ (Luminal B/HER2 positive); [4]TN (Triple negative); [5]HR (Hazard ratio); [6]CI (confidence interval)

4.5. Associations and concordance of the expression pattern of biomarkers

4.5.1. Estrogen receptor (ER)

In primary tumors (PT), ER positivity was found to be associated with early stages of the disease (TNM I and II, p=0.04) and with local relapse (p=0.025), whereas ER-negative mammary carcinomas were associated with lymphatic vessel invasion by neoplastic cells (p=0.011), Ki-67 index ≥14% (p=0.035), larger tumors (p=0.048), high-grade carcinomas (p=0.003), and with the presence of metastases in regional lymph nodes (p=0.038, supplementary Table 2). ER-negative relapse lesions were significantly associated with grade III tumors (p=0.02), with the presence of necrosis (p=0.007) and lymphatic invasion (p=0.041, supplementary Tables 3 to 5).

The overall concordance of ER status between the PT and LR was moderate (56.3%, 9/16), although smaller than between PT and RM (91.2%, 31/34) and between PT and DM (84.6%, 11/13). 40% (2/5) of the animals with ER-positive PT showed low expression levels of ER in metastases, in contrast with the low percentage of cats (6.5%, 2/31) which showed ER-negative mammary carcinomas coupled with one or more ER-positive metastases.

4.5.2. Progesterone receptor (PR)

The analysis of the expression pattern of PR revealed that 18.2% (4/22) of the animals with PR-positive mammary carcinomas showed PR-negative metastatic lesions, which was very similar to the 28.6% (4/14) of cats with PR-negative PT and PR-positive metastases. Further immunohistochemical analysis demonstrated that the majority of PR-positive primary and metastatic lesions were ER-negative. Indeed, 84.4% (27/32) of cats with PR-positive and ER-negative PT, 72.7% (8/11) showed PR-positive and ER-negative LR, 91.7% (22/24) had PR-positive and ER-negative RM, and 100% (7/7) showed PR-positive and ER-negative DM.

Mammary carcinomas with PR overexpression were significantly associated with neutered cats (p=0.049), while PR negative tumors were associated with CK5/6 negative status (p=0.034). Further, PR-positive recurrent lesions were significantly associated with the presence of necrotic areas (p=0.035, supplementary Tables 2 to 5).

The concordance of PR status in PT and LR was 68.8% (11/16), 82.4% (28/34) between PT and RM, and 61.5% (8/13) between PT and DM.

4.5.3. *f*HER2

Immunohistochemical analysis revealed that 31.1% (19/61) of the cats had *f*HER2-positive carcinomas, 42.9% (9/21) showed *f*HER2-positive relapses, 25% (12/48)

exhibited *f*HER2-positive regional metastases, and 26.1% (6/23) had *f*HER2-positive distant metastases (Table 18).

Slightly more than half of the animals with *f*HER2-positive mammary tumors had *f*HER2-negative metastatic lesions (54.5%, 6/11), contrasting with the small percentage of animals that showed *f*HER2-negative mammary carcinoma and *f*HER2-positive metastatic lesions (16%, 4/25). A concordance of 68.8% (11/16) was found for the *f*HER2 status between PT and LR, similarly to the concordance ratio between PT and RM (67.6%, 23/34), but lower than the concordance rate found between PT and DM (84.6%, 11/13). *f*HER2 did not present significant associations with the other clinicopathological features (supplementary Tables 2 to 5).

4.5.4. Cytokeratin 5/6 (CK5/6)

The percentage of CK5/6-positive samples was identical for primary tumors and metastases (Table 18). Indeed, 48.3% (29/61) of the cats with mammary carcinomas had CK5/6-positive tumors, 47.6% (10/21) had CK5/6-positive local relapses, 54.2% (26/48) showed CK5/6-positive regional metastases and 43.5% (10/23) had CK5/6-positive distant metastases.

Interestingly, when the animals that underwent surgery without chemotherapy were analyzed, the percentage of cats with CK5/6-negative mammary carcinoma that showed CK5/6-positive metastatic lesions was identical (33.3%, 6/18) to the percentage of cats that showed CK5/6-positive tumors and CK5/6-negative metastatic lesions (33.3%, 6/18).

Only PT showed significant associations between CK5/6 expression and clinicopathological features (supplementary Tables 6 to 9), with CK5/6-positive PT being associated with the presence of multiple mammary carcinomas (p=0.032), grade III tumors (Elston & Ellis classification, p=0.044) and PR-positive status (p=0.034), whereas CK5/6-negative carcinomas were associated with the absence of lymphatic vessel invasion (p=0.05). The concordance of results for CK5/6 status was of 56.3% between mammary carcinomas and LR (9/16), slightly lower than between the PT and RM (73.5%, 23/34), but identical to the concordance between PT and DM (53.8%, 7/13).

4.5.5. Ki-67 index

Analysis of Ki-67 expression levels showed a trend towards a higher mean throughout the disease progression, with primary tumors and local relapses displaying lower mean Ki-67 index (26.7 and 22.9%, respectively) than regional (32.2%) and distant metastases (36%). In fact, 77% (47/61) of PT and 71.4% (15/21) of RL showed a Ki-67

index ≥14%, whereas 83.3% (40/48) of RM and 91.3% (21/23) of DM also showed a Ki-67 index value equal or higher than 14%. We also observed a moderate concordance between the Ki-67 index of PT and LR (43.8%, 7/16) and high concordance rates between PT and RM (73.5%, 25/34), and between PT and DM (76.9%, 10/13). When a threshold value of 14% was used, 80% (8/10) of cats that showed Ki-67-negative mammary carcinoma had Ki-67-positive relapse/metastatic lesions, in contrast to only 15.4% (4/26) of animals that showed Ki-67 positive mammary tumor and Ki-67-negative relapse/metastatic lesions. Finally, mammary carcinomas with a Ki-67 index ≥14% showed a significant association with large tumor size (p=0.001), high-grade carcinomas (according to Mills *et al.* classification, p<0.0001), presence of necrotic areas (p=0.011) and with ER-negative expression (p=0.035, supplementary Tables 6 to 9).

4.5.6. Molecular subtypes

The molecular immunophenotyping of all mammary carcinomas (n=61), local relapses (n=21), regional (n=48), and distant metastases (n=23) was performed using the St Gallen's guidelines. Our results demonstrate that protein expression patterns could be different between PT, LR, RM, and DM, for the same animal. Indeed, luminal B (HER2− and HER2+) was the most common subtype in PT (52.5%; 32/61), while 65.2% (15/23) of DM were triple negative (basal and normal-like). Moreover, all cats with luminal A primary carcinomas (n=8) showed more aggressive subtypes in all metastatic lesions. Remarkably, the luminal B/HER2-positive subtype was well conserved between primary tumors and paired metastases (75%; 6/8), followed by the luminal B/HER2-negative subtype (63.6%; 7/11), by triple negative/basal-like subtype (50%; 2/4), by HER2-positive subtype (33.3%; 1/3) and by the triple negative normal-like subtype (25%; 1/4). Additional statistical analysis revealed that only LR showed significant associations with lymphatic vessel invasion by neoplastic cells (p=0.032, supplementary Tables 10 and 11). In fact, we found that triple negative subtypes were significantly associated with the presence of lymphatic invasion (p=0.032), while both luminal A and luminal B (HER2-negative and HER2-positive) tumors were associated with the absence of lymphatic vessel invasion in local relapses.

In summary, the maintenance of the tumor subtype through disease progression was relatively low, with a concordance rate of 31.3% (5/16) between the PT and the LR, of 47.1% (16/34) between the PT and the RM, and of 53.8% (7/11) between the PT and the DM.

5. Discussion

The post-mortem examination of 23 queens diagnosed with MC revealed that the lungs and/or pleura were the most common organs involved in metastatic disease (87%), followed by the non-regional lymph nodes (70%) and the liver (30.4%), which is similar to what is reported in humans (Goldhirsch *et al.*, 2013). At the moment of death, most of the animals had more than one organ invaded (87%, 20/23), thus revealing the high metastatic potential of this carcinoma type.

Our results also demonstrated that the majority of metastases showed loss of ER and PR expression compared to the corresponding primary tumors, as equally reported in patients with breast cancer (Simmons *et al.*, 2009; Aurilio *et al.*, 2014; Cummings *et al.*, 2014). This phenomenon was more evident for ER expression, with cats having ER-positive mammary tumors being associated with a better outcome, also as reported for women with breast cancer (Harris *et al.*, 2007; Hammond *et al.*, 2010; Kwast *et al.*, 2014). In addition, PR-positive mammary carcinomas were associated with neutered cats at the time of mastectomy. This can be explained by the fact that most of the animals were already spayed during adulthood, and were therefore exposed to the ovarian hormones most of their life, explaining the association found between these two parameters (de las Mulas *et al.*, 2000; Sorenmo *et al.*, 2013). Moreover, PR-positive LR were correlated with the presence of necrotic areas. Our data are consistent with findings reported for breast cancer patients, where, primary ER−/PR+ breast cancers exhibit a more aggressive behavior than ER+ or ER+/PR+ tumors (Yu *et al.*, 2008; Ng *et al.*, 2012).

Low concordance rates in *f*HER2 status were found between primary and relapse lesions (68.8%) and between primary tumors and regional metastases (67.6%), similarly to what is found in breast cancer patients (Chan *et al.*, 2012; Macfarlane *et al.*, 2012). This study also revealed that cats with CK5/6-positive mammary carcinomas were associated with poor prognostic features and lower overall survival time, as described for breast cancer patients (Livasy *et al.*, 2006; Bosch *et al.*, 2010; Joensuu *et al.*, 2013). Additionally, CK 5/6-positive mammary carcinomas were found to be correlated with the presence of multiple and poorly differentiated primary tumors, with PR-positive status, whereas CK 5/6-negative FMCs were associated with the absence of lymphatic vessel invasion.

Evaluation of Ki-67 expression showed that metastases had higher indexes than paired mammary carcinomas. Ki-67 expression was also associated with worse outcome features (larger tumor, high-grade carcinomas, presence of necrosis, and ER-negative expression), corroborating our data which significantly correlate lower OS rates with cats having high Ki-67 index mammary tumors, either in univariate or in multivariate

survival analysis, which is also reported for breast cancer patients (Yerushalmi *et al.*, 2010; Park *et al.*, 2012; Goldhirsch *et al.*, 2013; Pathmanathan *et al.*, 2013).
Finally, the luminal B/HER2-negative and the luminal B/HER2-positive were the most frequent subtypes out of the six evaluated, corroborating the data published by Brunetti *et al.* (2013). In parallel, the prevalence of the triple negative/basal-like FMC (16.4%) was lower than reported by Maniscalco *et al.* (2013) and Wiese *et al.* (2013), even though the frequency of the triple negative/basal-like metastases was higher (39.1%). These results are relevant as cats with basal-like mammary carcinomas are associated with lower OS and DFS periods, and larger tumors with lymphatic vessel invasion, as women with triple-negative/basal-like breast cancer (Bosch *et al.*, 2010). Moreover, molecular classification has proved to be an independent prognostic factor related to OS, with an animal with HER2-positive MC displaying a 438-fold increased risk of tumor-related death, in comparison with the other subtypes and the triple negative/basal-like mammary carcinomas presenting a hazard ratio of 210.8.
Altogether, our results disclose the high metastatic capacity of FMC and the heterogeneity of protein expression profiles between primary tumors and paired metastases. However, the majority of local relapses had less aggressive features when compared to paired primary tumor (smaller size, grade I tumors, absence of necrosis, and vascular invasion). From a molecular point of view, relapse lesions also showed a higher percentage of ER-positive cases, a lowest Ki-67 mean index and a low percentage of triple negative/ basal-like cases. We hypothesise that these findings could have resulted from a greater awareness and a closer follow-up of the animals before mastectomy, which enabled the early detection of disease relapses and, consequently, resulting in less aggressive characteristics.

6. Conclusions

Taking our results in consideration on the prognostic value of each molecular subtype, the FMC immunophenotyping should be routinely performed in order to choose the better therapeutic strategies, once FMC subtypes mimic breast cancer subtypes. The presence of metastases must also be carefully examined to improve the clinical management of the animal, considering that DM usually show a worse immunophenotype than matched primary tumor/local metastases probably due to an adaptive response to different microenvironments found in diverse tissues. Finally, the similarities found between FMC and human breast cancer highlight the fact that FMC can be used as a model for comparative oncology.

7. Supplemental material

Supplemental Table 1. Univariate survival analysis for ER status, PR status, HER2 status, CK 5/6 status and Ki-67 index levels in 42 female cats with mammary carcinoma.

	OS Mean (±SE)	OS Median (±SE)	*p*	DFS Mean (±SE)	DFS Median (±SE)	*p*
ER status			0.04*			0.524
Negative	480.1 (±77.1)	345 (±45.4)		277.9 (±44.9)	259 (±32)	
Positive	843.3 (±133.5)	488 (±276.22)		337.6 (±58.1)	315 (±117.4)	
PR status			0.92			0.62
Negative	568.1 (±120.5)	328 (±41.7)		254.7 (±55.4)	286 (±76.7)	
Positive	589.3 (±91)	479 (±71.3)		304.6 (±45.4)	266 (±24.3)	
***f*HER2 status**			0.414			0.324
Negative	599.3 (±93.4)	505 (±110.3)		308 (±42.6)	286 (±43.8)	
Positive	397.9 (±61.2)	345 (±112.5)		242.7 (±68.7)	209 (±38.3)	
CK 5/6			0.022*			0.781
Negative	736.3 (±117.3)	505 (±198.2)		310.8 (±45.1)	286 (±20.4)	
Positive	363 (±55.4)	325 (±63)		266.8 (±59.6)	243 (±147.1)	
Ki-67 index			0.011*			0.102
High	404.5 (±61.7)	328 (±29.6)		232.4 (±30)	266 (±21.6)	
Low	859.8 (±145.1)	716 (±204.4)		364.4 (±71.2)	340 (±118)	

Supplemental Table 2. Statistical association between the clinicopathological features of 61 cats with primary mammary carcinoma and ER, PR and *f*HER2 status.

Clinicopathological features	ER-negative (n=48)	ER-positive (n=13)	*p*	PR-negative (%) (n=29)	PR-positive (%) (n=32)	*p*	*f*HER2-negative (%) (n=42)	*f*HER2-positive (%) (n=19)	*p*
Age, years			0.073			0.232			0.608
< 10 years	7 (15.6%)	5 (38.5%)		7 (26.9%)	5 (15.6%)		8 (20.5%)	4 (21.1%)	
> 10 years	38 (84.4%)	8 (61.5%)		19 (73.1%)	27 (84.4%)		31 (79.5%)	15 (78.9%)	
Unknown†	3	0		3	0		3	0	
Breed			0.368			0.518			0.457
Inbreed	39 (81.2%)	10 (76.9%)		23 (79.3%)	26 (81.2%)		32 (76.2%)	17 (89.5%)	
Siamese	4 (8.3%)	1 (7.7%)		2 (6.9%)	3 (9.4%)		3 (7.1%)	2 (10.5%)	
Persian	2 (4.2%)	0 (0.0%)		2 (6.9%)	0 (0.0%)		2 (4.8%)	0 (0.0%)	
NFC[1]	3 (6.2%)	1 (7.7%)		2 (6.9%)	2 (6.2%)		4 (9.5%)	0 (0.0%)	
Russian Blue	0 (0.0%)	1 (7.7%)		0 (0.0%)	1 (3.1%)		1 (2.4%)	0 (0.0%)	
Spayed			0.256			0.049*			0.059
No	20 (42.6%)	7 (58.3%)		16 (59.3%)	11 (34.4%)		22 (53.7%)	5 (18.5%)	
Yes	27 (57.4%)	5 (41.7%)		11 (40.7%)	21 (65.6%)		19 (46.3%)	13 (72.2%)	
Unknown†	1	1		2	0		1	1	
Progestogens			0.545			0.317			0.437
No	15 (46.9%)	4 (36.4%)		8 (38.1%)	11 (50.0%)		14 (46.7%)	5 (38.5%)	
Yes	17 (53.1%)	7 (63.6%)		13 (61.9%)	11 (50.0%)		16 (53.3%)	8 (61.5%)	
Unknown†	16	2		8	10		12	6	
Multiple tumors			0.168			0.338			0.548
No	20 (41.7%)	8 (61.5%)		12 (41.4%)	16 (50.0%)		19 (45.2%)	9 (47.4%)	
Yes	28 (58.3%)	5 (38.5%)		17 (58.6%)	16 (50.0%)		23 (54.8%)	10 (52.6%)	
LNS[2]			0.09			0.098			0.186
Negative	7 (15.9%)	5 (38.5%)		3 (11.5%)	9 (29.0%)		10 (25.6%)	2 (11.1%)	
Positive	37 (84.1%)	8 (61.5%)		23 (88.5%)	22 (71.0%)		29 (74.4%)	16 (88.9%)	
Unknown†	4	0		3	1		3	1	
Stage			0.04*			0.062			0.793
I	2 (4.2%)	3 (23.1%)		2 (6.9%)	3 (9.4%)		3 (7.1%)	2 (10.5%)	
II	2 (4.2%)	2 (15.4%)		1 (3.4%)	3 (9.4%)		3 (7.1%)	1 (5.3%)	
III	31 (64.6%)	7 (53.8%)		15 (51.7%)	23 (71.9%)		25 (59.5%)	13 (68.4%)	
IV	13 (27.1%)	1 (7.7%)		11 (37.9%)	3 (9.4%)		11 (26.2%)	3 (15.8%)	

Tumor size			0.048*			0.417			0.545
<2 cm	8 (16.7%)	6 (46.2%)		7 (24.1%)	7 (21.9%)		8 (19.0%)	6 (31.6%)	
2-3 cm	19 (39.6%)	5 (38.5%)		9 (31.0%)	15 (46.9%)		17 (40.5%)	7 (36.8%)	
>3 cm	21 (43.8%)	2 (15.4%)		13 (44.8%)	10 (31.2%)		17 (40.5%)	6 (31.6%)	
Histopathology			0.327			0.465			0.226
Mucinous carcinoma	4 (8.3%)	1 (7.7%)		4 (13.8%)	1 (3.1%)		4 (9.5%)	1 (5.3%)	
TP[3] carcinoma	29 (60.4%)	11 (84.6%)		18 (62.1%)	22 (68.8%)		29 (69.0%)	11 (57.9%)	
Solid carcinoma	7 (14.6%)	1 (7.7%)		4 (13.8%)	4 (12.5%)		3 (7.1%)	5 (26.3%)	
Cribriform carcinoma	8 (16.7%)	0 (0.0%)		3 (10.3%)	5 (15.6%)		6 (14.3%)	2 (10.5%)	
MG[4] (EE[5] system)			0.107			0.502			0.175
I	0 (0.0%)	1 (7.7%)		1 (3.4%)	0 (0.0%)		0 (0.0%)	1 (5.3%)	
II	4 (8.3%)	2 (15.4%)		3 (10.3%)	3 (9.4%)		3 (7.1%)	3 (15.8%)	
III	44 (91.7%)	10 (76.9%)		25 (86.2%)	29 (90.6%)		39 (92.9%)	15 (78.9%)	
MG[4] (Mills *et al.*)			0.003*			0,587			0,678
I	3 (6.2%)	2 (15.4%)		1 (3.4%)	3 (9.4%)		2 (4.8%)	2 (10.5%)	
II	12 (25.0%)	9 (69.2%)		10 (34.5%)	12 (37.5%)		15 (35.7%)	7 (36.8%)	
III	33 (68.8%)	2 (15.4%)		18 (62.1%)	17 (53.1%)		25 (78.2%)	10 (52.7%)	
Necrosis			0.626			0.094			0.224
No	11 (22.9%)	3 (23.1%)		4 (13.8%)	10 (31.2%)		8 (19.0%)	6 (31.6%)	
Yes	37 (77.1%)	10 (76.9%)		25 (86.2%)	22 (68.8%)		34 (81.0%)	13 (68.4%)	
Lymphatic invasion			0.015*			0.109			0.17
No	32 (66.7%)	13 (100.0%)		24 (82.8%)	21 (65.6%)		33 (78.6%)	12 (63.2%)	
Yes	16 (33.3%)	0 (0.0%)		5 (17.2%)	11 (34.4%)		9 (21.4%)	7 (36.8%)	
Lymphocytic infiltration			0.57			0.315			0.533
No	9 (18.8%)	2 (15.4%)		4 (13.8%)	7 (21.9%)		8 (19.0%)	3 (15.8%)	
Yes	39 (81.2%)	11 (84.6%)		25 (86.2%)	25 (78.1%)		34 (81.0%)	16 (84.2%)	
Ulceration			0.641			0.303			0.376
No	40 (83.3%)	11 (84.6%)		23 (79.3%)	28 (87.5%)		36 (85.7%)	15 (78.9%)	
Yes	8 (16.7%)	2 (15.4%)		6 (20.7%)	4 (12.5%)		6 (14.3%)	4 (21.1%)	
ER status						0.204			0.163
Negative	-	-		21 (72.4%)	27 (84.4%)		35 (83.3%)	13 (68.4%)	
Positive	-	-		8 (27.6%)	5 (15.6%)		7 (16.7%)	6 (31.6%)	
PR status			0.204						0.602
Negative	21 (43.8%)	8 (61.5%)		-	-		20 (47.6%)	9 (47.4%)	
Positive	27 (56.2%)	5 (38.5%)		-	-		22 (52.4%)	10 (52.6%)	

***f*HER2 status**			0.163			0.602			
Negative	35 (72.9%)	7 (53.8%)		20 (69.0%)	22 (68.8%)		-	-	
Positive	13 (27.1%)	6 (46.2%)		9 (31.0%)	10 (31.2%)		-	-	
CK5/6 status			0.067			0.034*			0.175
Negative	22 (45.8%)	9 (75.0%)		19 (65.5%)	12 (38.7%)		19 (46.3%)	12 (63.2%)	
Positive	26 (54.2%)	3 (25.0%)		10 (34.5%)	19 (61.3%)		22 (53.7%)	7 (36.8%)	
Unknown†	0	1		0	1		1	0	
Ki-67 index levels			0.035*			0.242			0.455
Low	8 (16.7%)	6 (46.2%)		5 (17.2%)	9 (28.1%)		9 (21.4%)	5 (26.3%)	
High	40 (83.3%)	7 (53.8%)		24 (82.8%)	23 (71.9%)		33 (78.6%)	14 (73.7%)	
Local relapse			0.025*			0.09			0.286
No	35 (72.9%)	5 (38.5%)		22 (75.9%)	18 (56.2%)		29 (69.0%)	11 (57.9%)	
Yes	13 (27.1%)	8 (61.5%)		7 (24.1%)	14 (43.8%)		13 (31.0%)	18 (42.1%)	
Regional metastasis			0.038*			0.124			0.275
No	6 (13.0%)	5 (38.5%)		3 (10.7%)	8 (25.8%)		9 (22.0%)	2 (11.1%)	
Yes	40 (87.0%)	8 (61.5%)		25 (89.3%)	23 (74.2%)		32 (78.0%)	16 (88.9%)	
Unknown†	2	0		1	1		1	1	
Distant metastasis			0.831			0.583			0.75
No	1 (4.3%)	0 (0.0%)		0 (0.0%)	1 (7.1%)		1 (5.6%)	0 (0.0%)	
Yes	22 (95.7%)	1 (100.0%)		10 (100.0%)	13 (92.9%)		17 (94.4%)	6 (100.0%)	
Unknown†	25	12		19	18		24	13	

† Not considered in the calculation of the percentages neither in the statistical analysis. Abbreviations: [1]NFC, Norwegian forest cat; [2]LNS, Lymph node status; [3]TP, Tubulopapillary; [4]MG (malignancy grade); [5]EE (Elston & Ellis)

Supplemental Table 3. Associations between clinicopathological features and ER, PR and *f*HER2 status in local relapses of 16 female cats that were subjected only to mastectomy.

Clinicalpathological features	ER-negative (%) (n=8)	ER-positive (%) (n=8)	*p*	PR-negative (%) (n=7)	PR-positive (%) (n=9)	*p*	*f*HER2-negative (%) (n=10)	*f*HER2-positive (%) (n=6)	*p*
Tumor size			0.592			0.978			0.472
<2 cm	4 (50.0%)	5 (71.4%)		4 (57.2%)	5 (62.5%)		6 (60.0%)	3 (60.0%)	
2-3 cm	1 (12.5%)	1 (14.3%)		1 (14.2%)	1 (12.5%)		2 (20.0%)	0 (0.0%)	
>3 cm	3 (37.5%)	1 (14.3%)		2 (28.6%)	2 (25.0%)		2 (20.0%)	2 (40.0%)	
Unknown†	0	1		0	1		0	1	
Histopathology			0.107			0.46			0.295
TP[1] carcinoma	5 (62.5%)	2 (25.0%)		4 (57.2%)	3 (33.4%)		5 (50.0%)	2 (33.3%)	
Cribriform carcinoma	1 (12.5%)	2 (25.0%)		0 (0.0%)	2 (22.2%)		1 (10.0%)	1 (16.7%)	
Solid carcinoma	2 (25.0%)	0 (0.0%)		1 (14.2%)	2 (22.2%)		3 (30.0%)	0 (0.0%)	
Carcinoma “in situ”	0 (0.0%)	3 (37.5%)		2 (28.6%)	1 (11.1%)		1 (10.0%)	2 (33.3%)	
Squamous cell carcinoma	0 (0.0%)	1(12.5%)		0 (0.0%)	1 (11.1%)		0 (0.0%)	1 (16.7%)	
MG[2] (EE[3] system)			0.026*			0.109			0.317
I	0 (0.0%)	3 (37.5%)		2 (28.6%)	1 (11.1%)		1 (10.0%)	2 (33.3%)	
II	0 (0.0%)	2 (25.0%)		2 (28.6%)	0 (0.0%)		2 (20.0%)	0 (0.0%)	
III	8 (100.0%)	3 (37.5%)		3 (42.8%)	8 (88.9%)		7 (70.0%)	4 (66.7%)	
MG[2] (Mills *et al.* system)			0.014*			0.554			0.403
I	0 (0.0%)	4 (50.0%)		3 (42.8%)	1 (11.1%)		2 (20.0%)	2 (33.3%)	
II	2 (25.0%)	3 (37.5%)		2 (28.6%)	3 (33.3%)		3 (30.0%)	2 (33.3%)	
III	6 (75.0%)	1 (12.5%)		2 (28.6%)	5 (55.6%)		5 (50.0%)	2 (33.3%)	
Necrosis			0.007*			0.035*			1
No	0 (0.0%)	6 (75.0%)		5 (71.4%)	1 (11.1%)		4 (40.0%)	2 (33.3%)	
Yes	8(100.0%)	2 (25.0%)		2 (28.6%)	8 (88.9%)		6 (60.0%)	4 (66.7%)	
Lymphatic invasion			0.20			0.55			0.25
No	5 (62.5%)	8 (100.0%)		5 (71.4%)	8 (88.9%)		7 (70.0%)	6 (100%)	
Yes	3 (37.5%)	0 (0.0%)		2 (28.6%)	1 (11.1%)		3 (30.0%)	0 (0.0%)	
Lymphocytic infiltration			0.315			1			0.25
No	2 (25.0%)	5 (62.5%)		3 (42.8%)	4 (44.4%)		7 (70.0%)	6 (100%)	
Yes	6 (75.0%)	3 (37.5%)		4 (57.2%)	5 (55.6%)		3 (30.0%)	0 (0.0%)	
Ulceration			-			-			-
No	8 (100%)	8 (100%)		7 (100%)	9 (100%)		10 (100%)	6 (100%)	
Yes	0 (0.0%)	0 (0.0%)		0 (0.0%)	0 (0.0%)		0 (0.0%)	0 (0.0%)	
ER status						0.315			1
Negative	-	-		2 (28.6%)	6 (66.7%)		5 (50.0%)	3 (50.0%)	
Positive	-	-		5 (71.4%)	3 (33.3%)		5 (50.0%)	3 (50.0%)	

PR status			0.315						0.145
Negative	2 (25.0%)	5 (62.5%)		-	-		6 (60.0%)	1 (16.7%)	
Positive	6 (75.0%)	3 (37.5%)		-	-		4 (40.0%)	5 (83.3%)	
***f*HER2 status**			1			0.145			
Negative	5 (62.5%)	5 (62.5%)		6 (85.8%)	4 (44.4%)		-	-	
Positive	3 (37.5%)	3 (37.5%)		1 (14.2%)	5 (55.6%)		-	-	
CK 5/6 status			1			1			0.302
Negative	6 (75.0%)	3 (37.5%)		4 (57.2%)	5 (55.6%)		7 (70.0%)	2 (33.3%)	
Positive	2 (25.0%)	5 (62.5%)		3 (42.8%)	4 (44.4%)		3 (30.0%)	4 (66.7%)	
Ki-67 index levels			0.315			1			0.299
Low	2 (25.0%)	5 (62.5%)		5 (71.4%)	6 (66.7%)		8 (80.0%)	3 (50.0%)	
High	6 (75.0%)	3 (37.5%)		2 (28.6%)	3 (33.3%)		2 (20.0%)	3 (50.0%)	

† Not considered in the calculation of the percentages neither in the statistical analysis. Abbreviations: [1]TP, Tubulopapillary; [4]MG (malignancy grade); [5]EE (Elston & Ellis)

Supplemental Table 4. Associations between protein markers (ER, PR, *f*HER2, CK 5/6 and Ki-67) in regional relapses of 34 female cats that were subjected to mastectomy without chemotherapy

Regional metastasis	ER-negative (%) (n=30)	ER-positive (%) (n=4)	*p*	PR-negative (%) (n=12)	PR-positive (%) (n=22)	*p*	*f*HER2-negative (%) (n=24)	*f*HER2-positive (%) (n=10)	*p*
ER status						0.602			0.564
Negative	-	-		10 (83.3%)	20 (90.9%)		22 (91.7%)	8 (80.0%)	
Positive	-	-		2 (16.7%)	2 (9.1%)		2 (8.3%)	2 (20.0%)	
PR status			0.602						1
Negative	10 (33.3%)	2 (50.0%)		-	-		9 (37.5%)	3 (30.0%)	
Positive	20 (66.7%)	2 (50.0%)		-	-		15 (62.5%)	7 (70.0%)	
***f*HER2 status**			0.564			1			
Negative	22 (73.3%)	2 (50.0%)		3 (25.0%)	15 (68.2%)		-	-	
Positive	8 (26.7%)	2 (50.0%)		9 (75.0%)	7 (31.8%)		-	-	
CK 5/6 status			1			1			0.276
Negative	13 (43.3%)	2 (50.0%)		5 (41.7%)	10 (45.5%)		9 (37.5%)	6 (60.0%)	
Positive	17 (56.7%)	2 (50.0%)		7 (58.3%)	12 (54.5%)		15 (62.5%)	4 (40.0%)	
Ki-67 index levels			0.559			1			0.328
Low	5 (16.7%)	1 (25.0%)		2 (16.7%)	4 (18.2%)		3 (12.5%)	3 (30.0%)	
High	25 (83.3%)	3 (75.0%)		10 (83.3%)	18 (81.8%)		21 (87.5%)	7 (70.0%)	

Supplemental Table 5. Associations between protein markers (ER, PR, *f*HER2, CK 5/6 and Ki-67) in systemic relapses of 13 female cats that underwent only to mastectomy

Distant metastasis	ER-negative (%) (n=12)	ER-positive (%) (n=1)	*p*	PR-negative (%) (n=8)	PR-positive (%) (n=5)	*p*	*f*HER2-negative (%) (n=9)	*f*HER2-positive (%) (n=4)	*p*
ER status						1			0.308
Negative	-	-		7 (87.5%)	5 (100%)		9 (100%)	3 (75.0%)	
Positive	-	-		1 (12.5%)	0 (0.0%)		0 (0.0%)	1 (25.0%)	
PR status			1						0.217
Negative	7 (58.3%)	1 (100%)		-	-		7 (77.8%)	1 (25.0%)	
Positive	5 (41.7%)	0 (0.0%)		-	-		2 (22.2%)	3 (75.0%)	
***f*HER2 status**			0.308			0.217			
Negative	9 (75.0%)	0 (0.0%)		7 (87.5%)	2 (40.0%)		-	-	
Positive	3 (25.0%)	1 (100%)		1 (12.5%)	3 (60.0%)		-	-	
CK 5/6 status			1			1			1
Negative	9 (75.0%)	1 (100%)		6 (75.0%)	4 (80.0%)		7 (77.8%)	3 (75.0%)	
Positive	3 (25.0%)	0 (0.0%)		2 (25.0%)	1 (20.0%)		2 (22.2%)	1 (25.0%)	
Ki-67 index levels			0.154			0.487			1
Low	1 (8.3%)	1 (100%)		2 (25.0%)	0 (0.0%)		1 (11.1%)	1 (25.0%)	
High	11 (91.7%)	0 (0.0%)		6 (75.0%)	5 (100%)		8 (88.9%)	3 (75.0%)	

Supplemental Table 6. Statistical association between the clinicopathological features of 61 cats with primary mammary carcinoma and CK5/6 status and Ki-67 index levels.

Clinicopathological features	CK 5/6-negative (%) (n=31)	CK 5/6-positive (%) (n=29)	*p*	Ki-67 low levels (%) (n=14)	Ki-67 high levels (%) (n=47)	*p*
Age, years			0.474			0.603
< 10 years	5 (17.2%)	6 (21.4%)		3 (21.4%)	9 (20.5%)	
> 10 years	24 (82.8%)	22 (78.6%)		11 (78.6%)	35 (79.5%)	
Unknown†	2	1		0	3	
Breed			0.076			0.47
Inbreed	28 (90.3%)	20 (69.0%)		10 (71.4%)	39 (83.0%)	
Siamese	3 (9.7%)	2 (6.9%)		2 (14.3%)	3 (6.4%)	
Persian	0 (0.0%)	2 (6.9%)		0 (0.0%)	2 (4.3%)	
NFC[1]	0 (0.0%)	4 (13.8%)		2 (14.3%)	2 (4.3%)	
Russian Blue	0 (0.0%)	1 (3.4%)		0 (0.0%)	1 (2.1%)	
Spayed			0.389			0.164
No	15 (50.0%)	12 (42.9%)		8 (61.5%)	19 (41.3%)	
Yes	15 (50.0%)	16 (57.1%)		5 (38.5%)	27 (58.7%)	
Unknown†	1	1		1	1	
Progestogens			0.107			0.473
No	7 (33.3%)	12 (57.1%)		5 (50.0%)	14 (42.4%)	
Yes	14 (66.7%)	19 (42.9%)		5 (50.0%)	19 (57.6%)	
Unknown†	10	8		4	14	
Multiple tumors			0.032*			0.256
No	18 (58.1%)	9 (31.0%)		8 (57.1%)	20 (42.6%)	
Yes	13 (41.9%)	20 (69.0%)		6 (42.9%)	27 (57.4%)	
LNS[2]			0.251			0.122
Negative	4 (14.3%)	7 (25.0%)		5 (35.7%)	7 (16.3%)	
Positive	24 (85.7%)	21 (75.0%)		9 (64.3%)	36 (83.7%)	
Unknown†	3	1		0	4	
Stage			0.45			0.207
I	3 (9.7%)	1 (3.4%)		3 (21.4%)	2 (4.3%)	
II	1 (3.2%)	3 (10.3%)		1 (7.1%)	3 (6.4%)	
III	21 (67.7%)	17 (58.6%)		8 (57.1%)	30 (63.8%)	
IV	6 (19.4%)	8 (27.6%)		2 (14.3%)	12 (25.5%)	
Tumor size			0.11			0.001*
<2 cm	10 (32.3%)	3 (10.3%)		8 (57.1%)	6 (12.8%)	
2-3 cm	10 (32.3%)	14 (48.3%)		5 (35.7%)	19 (40.4%)	
>3 cm	11 (35.5%)	12 (41.4%)		1 (7.1%)	22 (46.8%)	

Histopathology			0.883			0.481
Mucinous carcinoma	2 (6.5%)	3 (10.3%)		0 (0.0%)	5 (10.6%)	
Tubulopapillary carcinoma	20 (64.5%)	19 (65.5%)		9 (64.3%)	31 (66.0%)	
Solid carcinoma	4 (12.9%)	4 (13.8%)		2 (14.3%)	6 (12.8%)	
Cribriform carcinoma	5 (16.1%)	3 (10.3%)		3 (21.4%)	5 (10.6%)	
MG[3] (EE[4] system)			0.044*			0.14
I	1 (3.2%)	0 (0.0%)		1 (7.1%)	0 (0.0%)	
II	5 (16.1%)	0 (0.0%)		2 (14.3%)	4 (8.5%)	
III	25 (80.6%)	29 (100%)		11 (78.6%)	43 (91.5%)	
MG[3] (Mills *et al.* system)			0.112			0.0001*
I	3 (9.7%)	0 (0.0%)		3 (21.4%)	1 (2.1%)	
II	13 (41.9%)	9 (31.0%)		10 (71.4%)	12 (25.5%)	
III	15 (48.4%)	20 (69.0%)		1 (7.2%)	34 (72.4%)	
Necrosis			0.132			0.011*
No	9 (29.0%)	4 (13.8%)		7 (50.0%)	7 (14.9%)	
Yes	22 (71.0%)	25 (86.2%)		7 (50.0%)	40 (85.1%)	
Lymphatic invasion			0.05			0.213
No	26 (83.9%)	18 (62.1%)		12 (85.7%)	33 (70.2%)	
Yes	5 (16.1%)	11 (37.9%)		2 (14.3%)	14 (29.8%)	
Lymphocytic infiltration			0.41			0.512
No	6 (19.4%)	4 (13.8%)		2 (14.3%)	9 (19.1%)	
Yes	25 (80.6%)	25 (86.2%)		12 (85.7%)	38 (80.9%)	
Ulceration			0.124			0.414
No	28 (90.3%)	22 (75.9%)		11 (78.6%)	40 (85.1%)	
Yes	3 (9.7%)	7 (24.1%)		2 (21.4%)	7 (14.9%)	
ER status			0.067			0.035*
Negative	22 (71.0%)	26 (89.7%)		8 (57.1%)	40 (85.1%)	
Positive	9 (29.0%)	3 (10.3%)		6 (42.9%)	7 (14.9%)	
PR status			0.034*			0.242
Negative	19 (61.3%)	10 (34.5%)		5 (35.7%)	24 (51.1%)	
Positive	12 (38.7%)	19 (65.5%)		9 (64.3%)	23 (48.9%)	
***f*HER2 status**			0.175			0.455
Negative	19 (61.3%)	22 (75.9%)		9 (64.3%)	33 (70.2%)	
Positive	12 (38.7%)	7 (24.1%)		5 (35.7%)	14 (29.8%)	
CK 5/6 status						0.220
Negative	-	-		9 (64.3%)	22 (47.8%)	
Positive	-	-		5 (35.7%)	24 (52.2%)	
Unknown†				0	1	

Ki-67 index levels			0.220			
Low	9 (29.0%)	5 (17.2%)		-	-	
High	22 (71.0%)	24 (82.8%)		-	-	
Unknown†	0	0				
Local relapse			0.464			0.327
No	20 (64.5%)	20 (69.0%)		8 (57.1%)	32 (68.1%)	
Yes	11 (35.5%)	9 (31.0%)		6 (42.9%)	15 (31.9%)	
Regional metastasis			0.149			0.073
No	3 (10.3%)	7 (24.1%)		5 (35.7%)	6 (13.3%)	
Yes	26 (89.7%)	22 (75.9%)		9 (64.3%)	39 (86.7%)	
Unknown†	2	0		0	2	
Distant metastasis			0.583			0.875
No	0 (0.0%)	1 (7.1%)		0 (0.0%)	1 (4.8%)	
Yes	10 (100%)	13 (92.9%)		3 (100%)	20 (95.2%)	
Unknown†	21	15		11	26	

† Not considered in the calculation of the percentages neither in the statistical analysis. Abbreviations: [1]NFC, Norwegian forest cat; [2]LNS, Lymph node status; [3]MG (malignancy grade); [4]EE (Elston & Ellis[25])

Supplemental Table 7. Statistical associations between clinicopathological features and the biomarkers CK 5/6 and Ki-67 index in local relapses of 16 female cats.

Clinicopathological features	CK 5/6-negative (%) (n=9)	CK 5/6-positive (%) (n=7)	*p*	Ki-67 low levels (%) (n=5)	Ki-67 high levels (%) (n=11)	*p*
Tumor size			0.455			0.721
<2 cm	5 (55.6%)	4 (66.7%)		2 (40.0%)	2 (18.2%)	
2-3 cm	2 (22.2%)	0 (0.0%)		3 (60.0%)	2 (18.2%)	
>3 cm	2 (22.2%)	2 (33.3%)		0 (0.0%)	7 (63.6%)	
Unknown†	0	1		0	0	
Histopathology			0.46			0.417
Tubulopapillary carcinoma	4 (44.4%)	3 (42.9%)		2 (40.0%)	5 (45.5%)	
Cribriform carcinoma	2 (22.2%)	1 (14.3%)		0 (0.0%)	3 (27.3%)	
Solid carcinoma	2 (22.2%)	0 (0.0%)		1 (20.0%)	1 (9.1%)	
Carcinoma “in situ”	1 (11.2%)	2 (28.5%)		1 (20.0%)	2 (18.2%)	
Squamous cell carcinoma	0 (0.0%)	1 (14.3%)		1 (20.0%)	0 (0.0%)	
MG[1] (EE[2] system)			0.331			0.592
I	1 (11.2%)	2 (28.5%)		1 (20.0%)	2 (18.2%)	
II	2 (22.2%)	0 (0.0%)		0 (0.0%)	2 (18.2%)	
III	6 (66.6%)	5 (71.5%)		4 (80.0%)	7 (63.6%)	
MG[1] (Mills *et al.* system)			0.255			0.56
I	1 (11.2%)	3 (42.9%)		2 (40.0%)	2 (18.2%)	
II	4 (44.4%)	1 (14.2%)		3 (60.0%)	2 (18.2%)	
III	4 (44.4%)	3 (42.9%)		0 (0.0%)	7 (63.6%)	
Necrosis			1			1
No	3 (33.3%)	3 (42.9%)		2 (40.0%)	4 (36.4%)	
Yes	6 (66.7%)	4 (57.1%)		3 (60.0%)	7 (63.6%)	
Lymphatic invasion			1			0.509
No	7 (77.8%)	6 (85.8%)		5 (100%)	8 (72.7%)	
Yes	2 (22.2%)	1 (14.2%)		0 (0.0%)	3 (27.3%)	
Lymphocytic infiltration			1			1
No	4 (44.4%)	3 (42.9%)		2 (40.0%)	5 (45.5%)	
Yes	5 (55.6%)	4 (57.1%)		3 (60.0%)	6 (54.5%)	
Ulceration			-			-
No	9 (100%)	7 (100%)		5 (100%)	11 (100%)	
Yes	0 (0.0%)	0 (0.0%)		0 (0.0%)	0 (0.0%)	

ER status			0.315			1
Negative	6 (66.7%)	2 (28.5%)		2 (40.0%)	6 (54.5%)	
Positive	3 (33.3%)	5 (71.5%)		3 (60.0%)	5 (45.5%)	
PR status			1			1
Negative	4 (44.4%)	3 (42.9%)		2 (40.0%)	5 (45.5%)	
Positive	5 (55.6%)	4 (57.1%)		3 (60.0%)	6 (54.5%)	
†HER2 status			0.302			0.299
Negative	7 (77.8%)	3 (42.9%)		2 (40.0%)	8 (72.7%)	
Positive	2 (22.2%)	4 (57.1%)		3 (60.0%)	3 (27.3%)	
CK 5/6 status						0.596
Negative	-	-		2 (40.0%)	7 (63.6%)	
Positive	-	-		3 (60.0%)	4 (36.4%)	
Ki-67 index levels			0.596			
Low	7 (77.8%)	4 (57.1%)		-	-	
High	2 (22.2%)	3 (42.9%)		-	-	

† Not considered in the calculation of the percentages neither in the statistical analysis. Abbreviations: [1]MG (malignancy grade); [2]EE (Elston & Ellis)

Supplemental Table 8. Statistical associations between protein markers (ER, PR, *f*HER2, CK 5/6 and Ki-67) in regional relapses of 34 female cats.

Regional relapses	CK 5/6-negative (%) (n=15)	CK 5/6-positive (%) (n=19)	*p*	Ki-67 low levels (%) (n=6)	Ki-67 high levels (%) (n=28)	*p*
ER status			1			0.559
Negative	13 (86.7%)	17 (89.5%)		5 (83.3%)	25 (89.3%)	
Positive	2 (13.3%)	2 (10.5%)		1 (16.7%)	3 (10.7%)	
PR status			1			1
Negative	5 (33.3%)	7 (36.8%)		2 (33.3%)	10 (35.7%)	
Positive	10 (66.7%)	12 (63.2%)		4 (66.7%)	18 (64.3%)	
***f*HER2 status**			0.276			0.328
Negative	9 (60.0%)	15 (78.9%)		3 (50.0%)	21 (75.0%)	
Positive	6 (40.0%)	4 (21.1%)		3 (50.0%)	7 (25.0%)	
CK 5/6 status						1
Negative	-	-		3 (50.0%)	12 (42.9%)	
Positive	-	-		3 (50.0%)	16 (57.1%)	
Ki-67 index levels			1			
Low	3 (20.0%)	3 (15.8%)		-	-	
High	12 (80.0%)	16 (84.2%)		-	-	

Supplemental Table 9. Statistical associations between protein markers (ER, PR, *f*HER2, CK 5/6 and Ki-67) in systemic relapses of 13 female cats.

Systemic relapses	CK 5/6-negative (%) (n=10)	CK 5/6-positive (%) (n=3)	*p*	Ki-67 low levels (%) (n=2)	Ki-67 high levels (%) (n=11)	*p*
ER status			1			0.154
Negative	9 (90.0%)	3 (100%)		1 (50.0%)	11 (100%)	
Positive	1 (10.0%)	0 (0.0%)		1 (50.0%)	0 (0.0%)	
PR status			1			0.487
Negative	6 (60.0%)	2 (66.7%)		2 (100%)	6 (54.5%)	
Positive	4 (40.0%)	1 (33.3%)		0 (0.0%)	5 (45.5%)	
***f*HER2 status**			1			1
Negative	7 (70.0%)	2 (66.7%)		1 (50.0%)	8 (72.7%)	
Positive	3 (30.0%)	1 (33.3%)		1 (50.0%)	3 (27.3%)	
CK 5/6 status						1
Negative	-	-		2 (100%)	8 (72.7%)	
Positive	-	-		0 (0.0%)	3 (27.3%)	
Ki-67 index levels			1			
Low	2 (20.0%)	0 (0.0%)		-	-	
High	8 (80.0%)	3 (100%)		-	-	

Supplemental Table 10. Statistical associations between clinicopathological features and molecular classification in primary tumors of 61 female cats.

Clinical features	Luminal A (%) (n=8)	LB/ HER2- (%) (n=18)	LB/ HER2+ (%) (n=14)	HER2+ (%) (n=4)	TN-basal like (%) (n=10)	TN-normal like (%) (n=7)	*p*
Age, years							
< 10 years	2 (25.0%)	4 (22.2%)	2 (14.3%)	1 (25.0%)	3 (33.3%)	0 (0.0%)	0.747
> 10 years	6 (75.0%)	14 (77.8%)	12 (85.7%)	3 (75.0%)	6 (66.7%)	5 (100%)	
Unknown†	0	0	0	0	1	2	
Breed							
Inbreed	6 (75.0%)	15 (83.3%)	12 (85.7%)	4 (100%)	6 (60.0%)	6 (85.7%)	
Siamese	1 (12.5%)	1 (5.6%)	2 (14.3%)	0 (0.0%)	0 (0.0%)	1 (14.3%)	0.415
Persian	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	2 (20.0%)	0 (0.0%)	
NFC[1]	1 (12.5%)	1 (5.6%)	0 (0.0%)	0 (0.0%)	2 (20.0%)	0 (0.0%)	
Russian Blue	0 (0.0%)	1 (5.6%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	
Spayed							
No	6 (75.0%)	6 (33.3%)	5 (38.5%)	0 (0.0%)	5 (55.6%)	5 (71.4%)	
Yes	2 (25.0%)	12 (66.7%)	8 (61.5%)	4 (100%)	4 (44.4%)	2 (28.6%)	0.083
Unknown†	0	0	1	0	1	0	
Progestogens							0.981
No	2 (33.3%)	7 (50.0%)	4 (44.4%)	1 (33.3%)	3 (50.0%)	2 (40.0%)	
Yes	4 (66.7%)	7 (50.0%)	5 (55.6%)	2 (66.7%)	3 (50.0%)	3 (60.0%)	
Unknown†	2	4	5	1	4	2	
Multiple tumors							0.701
No	5 (62.5%)	9 (50.0%)	7 (50.0%)	2 (50.0%)	3 (30.0%)	2 (28.6%)	
Yes	3 (37.5%)	9 (50.0%)	7 (50.0%)	2 (50.0%)	7 (70.0%)	5 (71.4%)	
LNS[2]							
Negative	3 (37.5%)	6 (35.3%)	2 (14.3%)	0 (0.0%)	1 (10.0%)	0 (0.0%)	
Positive	5 (62.5%)	11 (64.7%)	12 (85.7%)	3 (100%)	9 (90.0%)	6 (100%)	0.244
Unknown†	0	1	0	1	0	1	
Stage							
I	1 (12.5%)	2 (11.1%)	2 (14.3%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	
II	1 (12.5%)	2 (11.1%)	0 (0.0%)	1 (25.0%)	0 (0.0%)	0 (0.0%)	0.078
III	5 (62.5%)	13 (72.2%)	13 (72.2%)	3 (75.0%)	4 (40.0%)	3 (42.9%)	
IV	1 (12.5%)	1 (5.6%)	2 (14.3%)	0 (0.0%)	6 (60.0%)	4 (57.1%)	
Tumor size							0.301
<2 cm	4 (50.0%)	2 (11.1%)	5 (35.7%)	1 (25.0%)	1 (10.0%)	1 (14.3%)	
2-3 cm	3 (37.5%)	9 (50.0%)	5 (35.7%)	2 (50.0%)	4 (40.0%)	1 (14.3%)	
>3 cm	1 (12.5%)	7 (38.9%)	4 (28.6%)	1 (25.0%)	5 (50.0%)	5 (71.4%)	

Histopathology (carcinoma)							0.451
Mucinous	0 (0.0%)	2 (11.1%)	0 (0.0%)	1 (25.0%)	2 (20.0%)	0 (0.0%)	
Tubulopapillary	6 (75.0%)	14 (77.8%)	8 (57.1%)	2 (50.0%)	6 (60.0%)	4 (57.1%)	
Solid	0 (0.0%)	1 (15.6%)	4 (28.6%)	1 (25.0%)	1 (10.0%)	1 (14.3%)	
Cribriform	2 (25.0%)	1 (15.6%)	2 (14.3%)	0 (0.0%)	1 (10.0%)	2 (28.6%)	
MG[3] (EE[4] system)							0.766
I	0 (0.0%)	0 (0.0%)	1 (7.1%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	
II	1 (12.5%)	1 (5.6%)	2 (14.3%)	1 (25.0%)	0 (0.0%)	1 (14.3%)	
III	7 (87.5%)	17 (94.4%)	11 (78.6%)	3 (75.0%)	10 (100%)	6 (85.7%)	
MG[3] (Mills *et al.* system)							0.114
I	1 (12.5%)	1 (5.6%)	2 (14.2%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	
II	6 (75.0%)	6 (33.3%)	6 (42.9%)	1 (25.0%)	3 (30.0%)	0 (0.0%)	
III	1 (12.5%)	11 (61.1%)	6 (42.9%)	3 (75.0%)	7 (70.0%)	7 (100%)	
Necrosis							0.468
No	2 (25.0%)	5 (27.8%)	5 (35.7%)	1 (25.0%)	1 (10.0%)	0 (0.0%)	
Yes	6 (75.0%)	13 (72.2%)	9 (64.3%)	3 (75.0%)	9 (90.0%)	7 (100%)	
Lymphatic invasion							0.676
No	7 (87.5%)	14 (77.8%)	8 (57.1%)	3 (75.0%)	8 (80.0%)	5 (71.4%)	
Yes	1 (12.5%)	4 (22.2%)	6 (42.9%)	1 (25.0%)	2 (20.0%)	2 (28.6%)	
Lymphocytic infiltration							0.266
No	0 (0.0%)	6 (33.3%)	2 (14.3%)	1 (25.0%)	2 (20.0%)	0 (0.0%)	
Yes	8 (100%)	12 (66.7%)	12 (85.7%)	3 (75.0%)	8 (80.0%)	7 (100%)	
Ulceration							0.581
No	6 (75.0%)	16 (88.9%)	12 (85.7%)	3 (75.0%)	7 (70.0%)	7 (100%)	
Yes	2 (25.0%)	2 (11.1%)	2 (14.3%)	1 (25.0%)	3 (30.0%)	0 (0.0%)	
Local relapse							0.254
No	4 (50.0%)	11 (61.1%)	7 (50.0%)	3 (75.0%)	9 (90.0%)	6 (85.7%)	
Yes	4 (50.0%)	7 (38.9%)	7 (50.0%)	1 (25.0%)	1 (10.0%)	1 (14.3%)	
Regional metastasis							0.293
No	3 (37.5%)	5 (29.4%)	2 (14.3%)	0 (0.0%)	1 (10.0%)	0 (0.0%)	
Yes	5 (62.5%)	12 (70.6%)	12 (85.7%)	3 (100%)	9 (90.0%)	7 (100%)	
Unknown†	0	1	0	1	0	0	
Distant metastasis							0.411
No	0 (0.0%)	1 (20.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	
Yes	3 (100%)	4 (80.0%)	6 (100%)	0 (0.0%)	5 (100%)	5 (100%)	
Unknown†	5	13	8	4	5	2	

† Not considered in the calculation of the percentages neither in the statistical analysis. Abbreviations: [1]NFC, Norwegian forest cat; [2]LNS, Lymph node status; [3]MG (malignancy grade); [4]EE (Elston & Ellis)

Supplemental Table 11. Statistical associations between clinicopathological features and molecular classification in relapse tumors of 16 female cats.

Clinicopathological features	Luminal A (%) (n=2)	Luminal B/HER2- (%) (n=6)	Luminal B/HER2+ (%) (n=6)	TN-basal like (%) (n=1)	TN-normal like (%) (n=1)	*p*
Tumor size						0.186
<2 cm	1 (50.0%)	5 (83.3%)	3 (60.0%)	0 (0.0%)	0 (0.0%)	
2-3 cm	1 (50.0%)	1 (16.7%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	
>3 cm	0 (0.0%)	0 (0.0%)	2 (40.0%)	1 (100%)	1 (100%)	
Unknown†	0	0	1	0	0	
Histopathology (carcinoma)						0.685
Tubulopapillary	2 (100%)	1 (16.7%)	2 (33.3%)	1 (100%)	1 (100%)	
Cribriform	0 (0.0%)	3 (50.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	
Solid	0 (0.0%)	1 (16.7%)	1 (16.7%)	0 (0.0%)	0 (0.0%)	
"in situ"	0 (0.0%)	1 (16.7%)	2 (33.3%)	0 (0.0%)	0 (0.0%)	
Squamous cell	0 (0.0%)	0 (0.0%)	1 (16.7%)	0 (0.0%)	0 (0.0%)	
MG[1] (EE[2] system)						0.685
I	0 (0.0%)	1 (16.7%)	2 (33.3%)	0 (0.0%)	0 (0.0%)	
II	0 (0.0%)	2 (33.3%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	
III	2 (100%)	3 (50.0%)	4 (66.7%)	1 (100%)	1 (100%)	
MG[1] (Mills *et al.* system)						0.787
I	1 (50.0%)	1 (16.7%)	2 (33.3%)	0 (0.0%)	0 (0.0%)	
II	1 (50.0%)	2 (33.3%)	2 (33.3%)	0 (0.0%)	0 (0.0%)	
III	0 (0.0%)	3 (50.0%)	2 (33.3%)	1 (100%)	1 (100%)	
Necrosis						0.777
No	1 (50.0%)	3 (50.0%)	2 (33.3%)	0 (0.0%)	0 (0.0%)	
Yes	1 (50.0%)	3 (50.0%)	4 (66.7%)	1 (100%)	1 (100%)	
Lymphatic invasion						0.032*
No	2 (100%)	5 (83.3%)	6 (100%)	0 (0.0%)	0 (0.0%)	
Yes	0 (0.0%)	1 (16.7%)	0 (0.0%)	1 (100%)	1 (100%)	
Lymphocytic infiltration						0.386
No	0 (0.0%)	3 (50.0%)	4 (66.7%)	0 (0.0%)	0 (0.0%)	
Yes	2 (100%)	3 (50.0%)	2 (33.3%)	1 (100%)	1 (100%)	
Ulceration						-
No	2 (100%)	6 (100%)	6 (100%)	1 (100%)	1 (100%)	
Yes	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	

† Not considered in the calculation of the percentages neither in the statistical analysis. Abbreviations: [1]MG (malignancy grade); [2]EE (Elston & Ellis)

Chapter V – Serum HER2 levels are increased in cats with mammary carcinomas and predict tissue HER2 status

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1. Abstract

HER2 is overexpressed in about 30% of feline mammary carcinomas (FMC) and in 15-30% of breast cancers. Women with HER2-positive breast tumors are associated with shorter survival. However therapies targeting HER2 are available and show increasing success. This study aimed to optimise the detection and quantification of serum HER2 (sHER2) in cat and to evaluate its potential in diagnosing cats with mammary carcinomas (MC) overexpressing HER2. A prospective study was conducted in 60 queens showing MC and 20 healthy animals. Pre-operative serum samples were collected for sHER2 quantification using an immunoassay routinely employed in humans (ELISA) and a cheaper immunoblot assay (Dot blot). sHER2 levels were compared with tissue HER2 status assessed by immunohistochemistry. Queens with FMC showed significantly higher mean levels of sHER2, both by ELISA and Dot blot assay. A significant difference was also found between sHER2 levels of cats with HER2-positive MC and those with low-expressing HER2 MC. Further, a significant correlation between sHER2 levels and tumor HER2 status was found, particularly when ELISA was applied ($r=0.58$, $p<0.0001$). The value of 10 ng/ml was proposed as the optimal cut-off for both immunoassays by ROC analysis. Like in humans, sHER2 levels are increased in cats with MC HER2-positive, strongly suggesting that evaluation of sHER2 levels can be very useful in feline oncology. The results obtained show that ELISA and dot blot assay can replace the immunohistochemistry technique, due to their efficacy and lower costs for diagnostic purposes and for monitoring the response to anti-HER2 therapies in cats.

Keywords: Feline mammary carcinomas; HER2; serum HER2 levels; ELISA; Dot blot

2. Introduction

Recently, advances in genomics and proteomics have greatly expanded our knowledge about molecular mechanisms involved in oncogenesis and cancer progression (Hanahan & Weinberg, 2000). Indeed, human breast cancer has evolved from a single disease to a disease with different molecular subtypes, distinct clinical characteristics and outcomes (Reis-Filho *et al.*, 2011; The Cancer Genome Atlas Network, 2012; Stephens *et al.*, 2012). Animal models have also been crucial in increasing our understanding of tumorigenesis, with mouse species being the most commonly used because their small size and short gestation period (Rowell *et al.*, 2011). However, due to several limitations in using laboratory rodents, prevailing tumors in pets appear to be good alternative models, especially because of their high incidence. Moreover, pets also share a similar environment to humans and show a more equivalent body size, thus facilitating the pharmacokinetic and toxicological studies and shortening the phase I trials in humans (Rowell *et al.*, 2011; Vail & MacEwen, 2000; Porrello *et al.*, 2006; Paoloni *et al.*, 2008; Airley *et al.*, 2012).

Feline mammary carcinomas (FMC) are very common in cats, showing an incidence that ranges between 12 to 40% of all tumors (Lana *et al.*, 2007; Vascellari *et al.*, 2009). Their epidemiological and histopathological features closely resemble those found in the more aggressive breast cancer types (De Maria *et al.*, 2005). Thus, it has been proposed as a putative model for cancer studies (Vail & MacEwen, 2000; De Maria *et al.*, 2005; Winston *et al.*, 2005; Ordás *et al.*, 2007; Burrai *et al.*, 2010; Santos *et al.*, 2012; Soares *et al.*, 2013b). Recently, some reports have shown that the feline homologue of human epidermal growth factor receptor-2 proto-oncogene (HER2) is overexpressed in 33%-60% of FMC (Ordás *et al.*, 2007; Soares *et al.*, 2013a; Millanta *et al.*, 2005a) and it was associated with a shorter overall survival (OS) as in human HER2 positive breast tumors (Millanta *et al.*, 2005a), even though the HER2 gene amplification could not be detected (Ordás *et al.*, 2007; Soares *et al.*, 2013a).

HER2 has a molecular mass of 185 kDa and is a transmembrane glycoprotein which comprises three domains: an extracellular domain (ECD), a short transmembrane region and an intracellular domain with tyrosine kinase activity (Coussens *et al.*, 1985; Shafiee *et al.*, 2013; Carney *et al.*, 2013). In women, the gold standard method to identify HER2-positive breast tumors is immunohistochemistry (IHC) and also fluorescence in situ hybridization (FISH), which has been used to identify HER2 gene amplification status in ambiguous cases (Harris *et al.*, 2007; Wolff *et al.*, 2013). Nevertheless, several limitations, like the impossibility of conducting continuous follow-up after the invasive surgery (considering that the status of the patient is dependent on the tumor biopsy or excision which cannot be performed in the metastatic patient), and the high costs of the reagents, led to the development of new non-invasive techniques to quantify HER2 in serum (sHER2). The HER2-ECD is shed from the surface of tumor cells into the bloodstream via protease activity (A Disintegrin And Metalloproteinase domain-containing proteins - ADAM family), allowing its detection in sera by ELISA or by a chemiluminescence method (Kong *et al.*, 2012). In fact, many studies have found that breast cancer patients with elevated HER2-ECD levels were

associated with higher relapse rates and worse prognosis (Esteva *et al.*, 2005; Ludovini *et al.*, 2008; Kong *et al.*, 2012). More recently, it was reported that the Dot blot assay could also be used to measure sHER2 levels, being a less expensive method and representing a good alternative to closely follow the tumor disease progression (Tan *et al.*, 2011; Kong *et al.*, 2012; Lam *et al.*, 2012). To the best of our knowledge, no studies have been published on the utility of assessing sHER2 levels in small animals. Thus, the main objectives of this study were: i) to accurately quantify the sHER2 levels in cats using both ELISA and the Dot-blot assay; ii) to evaluate the usefulness of measuring sHER2 levels in the diagnosis of FMC overexpressing HER2; and iii) to determine the optimal cut-off value for the ELISA and the dot blot assay, that would differentiate cats with mammary carcinoma overexpressing HER2 from cats with HER2-negative mammary carcinomas or healthy animals.

3. Material & Methods

3.1. Cat study population

Sixty female cats with spontaneous mammary carcinomas that underwent surgical treatment at the Small Animal Hospital of the Veterinary Medicine Faculty, University of Lisbon, were selected in a prospective study from June 2011 to September 2013. For each animal, the following clinicopathological features were recorded: age, breed, reproductive status, progestogens administration, number, location and size of tumor lesions, performed treatment (none, surgery, surgery plus chemotherapy), histopathological classification, malignancy grade, presence of tumor necrosis, lymphatic invasion by tumor cells, lymphocytic infiltration, cutaneous ulceration, regional lymph node involvement, stage of the disease (TNM system), DFS and OS (Misdorp *et al.*, 2002).
Excised mammary glands, mammary tumors and regional lymph nodes from the animals were immediately fixed in 10% formalin neutralized with 0.1 M phosphate buffer (pH 7.2), during a period no longer than 48 hours. All samples were embedded into paraffin blocks and serial histological sections of 3 μm thickness were prepared, prior to hematoxylin and eosin staining. Carcinomas are classified according to the WHO system adapted by Misdorp *et al.* (1999) and the degree of malignancy was assessed according to the Elston and Ellis grading system (Misdorp *et al.*, 1999; Elston & Ellis, 1998), which classifies tumors into grade I (well differentiated), grade II (moderately differentiated), and grade III (poorly differentiated).

3.2. Tissue HER2, ER and PR status and Ki-67 assessment

A representative area of each FMC with a diameter of 0.6 cm was selected and tissue sections of 3μm thickness were mounted on glass slides (Star Frost adhesive glass slides, Thermo Scientific, Rockford, USA), deparaffinized with xylene and hydrated in a graded ethanol series to distilled water. For HER2, ER and Ki-67 immunostaining, antigen retrieval was performed by immersing glass tissue slides in citrate buffer (0.01M $NaCH_3COO$, pH 6.0) and using a pressure cooker (2 min at 2 atm), while for PR immunodetection, an immersion in water bath (60 min at 95ºC) was

performed (Soares *et al.*, 2013a). Slides were cooled for 10 min at room temperature and rinsed twice for 5 min in triphosphate buffered saline (TBS). Endogenous peroxidase activity was blocked with Peroxidase Block Novocastra (Novocastra, Newcastle, UK) for 5 min. Tissue samples were then incubated at 4ºC overnight, in a humidified chamber, with the following primary antibodies: mouse anti-HER2 (clone CB11, 1:200, Invitrogen, Carlsbad, CA, USA), mouse anti-ER (clone 6F11, 1:125, Thermo Scientific), rabbit anti-PR (clone 1E2, ready-to-use, Ventana, Tucson, USA) and rabbit anti-Ki-67 (polyclonal, 1:500, Thermo Scientific). The staining was performed using a modified streptavidin-peroxidase conjugate method based on the poly-HRP anti-rabbit IgG detection system (Novolink MaxPolymer Detection System, Leica Biosystems, Wetzlar, Germany), following the manufacturer's guidelines. Finally, tissue sections were counterstained with Mayer's hematoxylin (Merck, New Jersey, USA). HER2 immunoreactivity was scored according to the American Society of Clinical Oncology's recommendations (Wolff *et al.*, 2013), as summarized in Table 22. Briefly, FMC were classified as HER2-negative when scored 0 or +1 and HER2-positive if scored as +2 or +3 (Santos *et al.*, 2013; Soares *et al.*, 2013a). Mammary carcinomas were also evaluated for ER/PR status using the Allred score system on a scale of 0 to 8 (Table 23), and only tumors with a score ≥ 2 were considered positive (Harvey *et al.*, 1999; Mohsin *et al.*, 2004; Hammond *et al.*, 2010). The Ki-67 proliferation index was determined by dividing the number of tumor cells showing positive nuclear immunostaining per 1000 tumor cells analyzed over at least three high-amplified microscopic fields (Soares *et al.*, 2016b). Tumors were considered highly proliferative when more than 14% of the neoplastic cells nuclei expressed Ki-67 (Cheang *et al.*, 2009; Soares *et al.*, 2016b).

Table 22. HER2 immunohistochemistry scoring criteria

Score	Interpretation
0	No staining
+1	Weak, incomplete membrane staining in any proportion of tumor cells
+2	Complete membrane staining that has either no uniform or is weak in intensity, but with obvious circumferential distribution in at least 10% of cells
+3	Uniform intense membrane staining of at least 10% of invasive tumor cells

Finally, histological samples of feline mammary carcinomas with previous known ER/PR/HER2 status were used as controls, whereas a feline tonsil tissue sample was used as a positive control for the assessment of Ki-67 index, according to the manufacturer's instructions.

All slides were independently subjected to blind scoring by two independent pathologists. Discordant interpretations were further debated and settled using a multiobserver microscope.

Table 23. IHC semi-quantitative scoring system for ER/PR assessment (Harvey *et al.*, 1999; Mohsin *et al.*, 2004)

Proportion of positive staining tumor cells		Average staining intensity	
Score	Interpretation	Score	Interpretation
0	No staining	0	None
1	<1%	1	Weak
2	1-10%	2	Average
3	10-33%	3	Strong
4	33-66%		
5	>66%		
Allred score (0-8) = proportion of positive staining tumor cells (0-5) + average staining intensity (0-3)			

3.3. Quantitative immunoassays to measure sHER2 levels

A blood sample was collected from all the sixty queens with mammary carcinoma and from twenty healthy queens presented for elective ovariohysterectomy. The serum was separated from clotted blood by centrifugation (1500 g, 10 min, 4ºC), stocked in small aliquots (100 µL) and stored at -80ºC.

Considering the extensive sequence homology between human HER2 receptor and its homologue in *Felis catus* (93%), sHER2 levels in cats were evaluated by using a commercial ELISA-based kit, suitable to measure sHER2 levels in humans, and also by an optimised Dot blot procedure. Measurements were performed in a blind manner, without knowing IHC results and discarding all feline blood samples that show hemolysis (n=13), as recommended for humans (Lippi *et al.*, 2012).

3.3.1. Enzyme-linked immunosorbent assay (ELISA)

Serum HER2 levels were measured in 67 cats (47 ill and 20 healthy) using an ELISA sandwich assay (sHER2 Platinum ELISA kit, eBioscience, San Diego, USA), following the manufacturer's recommendations. A standard curve was generated using seven solutions of recombinant human HER2-ECD (rHER2-ECD) with known concentrations (0.16, 0.31, 0.63, 1.25, 2.5, 5 and 10 ng/ml). Briefly, first row of a 96-well ELISA plate was coated with 100 µl/well of each rHER2-ECD dilution, in duplicate, on "standards wells", whereas 10 µl of each serum sample was added to 90 µl of assay buffer in "sample wells", also in duplicates. After two consecutive washes (2×300µl with Wash Buffer), 50 µl of an HRP-conjugated mouse anti-IgG was added to each well and incubated at 37ºC, for 2 hours, on a microplate shaker at 100 rpm. After a second washing step (3×300 µl), 100 µl of the 3,3′,5,5′-tetramethyl-benzidine (TMB) substrate solution was added to each well and the final mixture was incubated at RT, for 10 min, in the dark. The reaction was interrupted by adding 100 µl of stop solution per well and absorbance was measured by a spectrophotometer (LabSystems IEMS Reader MF, Labsystems/Thermo Scientific, Helsinki, Finland) using 450 nm as the primary wavelength and 620 nm as reference wavelength.

3.3.2. Dot blot assay

After an initial optimization using serum samples from healthy cats, two FMC cell lines with known HER2 expression status and three antibodies against HER2, detection and quantification of sHER2 levels were performed in the sera of 67 cats. Briefly, after drawing a 1 cm square grid on a nitrocellulose membrane (Protan – BA83, Whatman GmbH, Dassel, Germany) with a pencil, 1 µl of each serum sample was spotted at the center of each square, allowing the evaluation of 40 sera per membrane (8 cm x 6 cm). The membranes were then dried at 37ºC for an hour, before being washed and blocked with 0.1M Tris-buffered saline buffer containing 0.1% Tween 20 and 1% bovine serum albumin (TBST-BSA, pH 8.0), for 30 min at room temperature (RT). Then, blots were incubated during 90 min at RT, with an anti-HER2 antibody (clone SP3, dilution 1:1000, Zytomed, Berlin, Germany) that specifically recognizes the extracellular domain of HER2 (Ricardo *et al.*, 2007; Nassar *et al.*, 2009). Later, membranes were washed for 30 min with TBST (3 x 10 min) and incubated for 30 min at RT with an appropriate secondary antibody (1:100,000; goat anti-rabbit IgG horseradish peroxidase conjugated – HRP, Southern Biotech, Birmingham, USA). The presence of HER2 in serum was revealed by the use of an enhanced chemiluminescence detection kit (Clarity Western ECL Substrate, Bio-rad, California, USA), and a semi-quantitative analysis was performed by comparing the signal intensity obtained in serum dots to the signal intensities of crescent concentrations of rHER2-ECD (5, 10, 15, 25, 50, 75 and 100 ng/ml, eBioscience, San Diego, USA) diluted in 30% BSA-Tris-buffered saline plus 0.05% Tween 20 (TBST) and spotted in the first line of the nitrocellulose membrane. Serum HER2 levels were scored by two independent observers and, discordant interpretations were discussed to reach a consensus. All serum samples were evaluated in three independent experiments.

Finally, two antibodies raised against different regions of the intracellular domain of HER2 receptor were also used to determine whether this domain is present in the soluble HER2 forms, thus detectable in the serum of cats (clone A0485, dilution 1:3500, Dako Denmark A/S, Glostrup, Denmark and clone 4B5, dilution 1:20, Ventana Medical Systems Inc., Tucson, AZ, USA).

3.4. Western blotting analysis

To evaluate antibody specificity, two feline serum samples with distinct HER2 levels previously determined by the dot blot assay, were probed with the anti-HER2 monoclonal antibody raised against the ECD (clone SP3). The molecular weight of the sHER2 fragments were compared with the band of recombinant human HER2-ECD, and with the bands detected in whole cell extracts of a human HER2-overexpressing breast cancer cell line (SKBR3, ATCC, Manassas, Virginia, USA) and a feline HER2-positive mammary carcinoma cell line (FMCp, kindly provided by Prof. Nobuo Sasaki, Tokyo University, Japan).

SKBR3 and FMCp cells grown in 30mm dishes were washed three times with phosphate-buffered saline (PBS) and lysed in radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris, pH 8.2; 150 mM NaCl; 0,5% NP40; 0,5% sodium deoxycolate; 0,1% SDS) supplemented with the following

protease and phosphatase inhibitors: cOmplete, Mini, EDTA-free (Roche), Phosphatase Inhibitor Cocktail Set V, 50x (Calbiochem, San Diego, USA) and PhoStop (Roche, Basel, Switzerland). After lysis, whole cell extracts were boiled at 95ºC for 15 min, centrifuged at 14,000 g for 2 min at 4ºC and stored at -80ºC.

The diluted rHER2-ECD, the whole cell lysates and the serum samples were fractioned by 7.5% SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane with 0.2 µm pore diameter (Whatman Schleicher & Schuell, Whatman GmbH, Dassel, Germany). The immunoblot was initially blocked using 2.5% (w/v) bovine serum albumin (BSA, Sigma) in TBST, to inhibit non-specific binding. For immunoblot analysis, membranes were then incubated with the primary and secondary antibodies used in the Dot blot assay. Signal intensity of reactive bands was detected by autoradiography using an enhanced chemiluminescence detection kit (Luminata Crescendo, ECL detection system, Millipore, Darmstadt, Germany).

3.5. Statistical Analysis

All statistical analyzes were carried out using the Statistical Package for the Social Sciences for Windows software (SPSS, version 21.0, IBM, Armonk, New York, USA) and a two-tailed *p* value less than 0.05 was considered statistically significant.

After testing for normality, the Wilcoxon test was used to compare the sHER2 levels between cats with mammary carcinoma and healthy ones, and also to compare sHER2 levels and tissue HER2 status among cancer group cats. Receiver-operating characteristics (ROC) curves were performed to choose the optimal cut-off value for the ELISA and Dot blot assay, and to determine the sensitivity and specificity of both techniques using IHC as a gold standard technique. The concordance between the three assays was estimated by the Kappa test and correlations were evaluated by Spearman's rank correlation coefficient. The Fisher’s exact test was used to assess the associations between sHER2 levels/tumor HER2 status and the clinicopathological features (breed, reproductive status, progestogens administration, prescribed treatment, number and location of tumor lesions, primary tumor size, lymph node status, stage of disease, histopathological classification, malignancy grade, presence of necrotic areas within the neoplasia, lymphatic vessel invasion by tumor cells, lymphocytic infiltration, cutaneous ulceration, Ki-67 index, HER2, ER and PR status). Whenever a cat showed multiple mammary tumors, the carcinoma with higher HER2 score (assessed by IHC), was the one selected for further studies. In cats with two or more mammary carcinomas showing equal HER2 scores, the lesion with higher malignancy grade and size was selected for statistical studies, since these two features have previously been associated with poor prognosis (MacEwen *et al.*, 1984; Seixas *et al.*, 2011). Animals were also grouped by the tumor size, according to the TNM classification (<2 cm; 2-3 cm; >3 cm).

Overall survival (OS) period was defined as the time elapsed between the initial diagnosis and the death/euthanasia due to tumor metastasis. Disease-free survival (DFS) time was calculated from the date of surgery to the date of relapse (local, in other mammary gland or in distant organs) or

death from cancer-related causes. Survival curves were estimated using the Kaplan-Meier method and the Log-rank test was used to compare the outcome (OS median and DFS median), regarding sHER2 levels and tumor HER2 status. For OS analysis, animals that died from a disease unrelated to mammary tumors or were lost during the follow-up were excluded.

4. Results

4.1. Animal study population

A total of sixty queens displaying mammary carcinoma were used in the study. Their main clinicopathological characteristics are summarized in Table 24. The mean age at diagnosis was 11.57 years (range, 7-17 years) and all animals were treated surgically, except one, which displayed pulmonary metastasis. Fifty (83.3%) cats were subjected to unilateral mastectomy, six (10%) to bilateral mastectomy and the remaining 3 (6.7%) to regional mastectomy.

After surgery, five of these queens were subjected to anthracycline-based adjuvant chemotherapy (doxorubicin, 25 mg/m^2, intravenously, every 3 weeks for 5 cycles). Tumor samples from the untreated animal were collected after euthanasia.

The mean size of the primary mammary carcinomas was 2.33 cm (range 0.3-7 cm) and the mean value of Ki-67 proliferation index was 19.5% (ranging between 1.2% and 46%). Twenty two (36.7%) cats showed HER2-overexpressing MC (assessed by IHC), out of which nine (15%) were classified with a 3+ and thirteen (21.7%) with a 2+ score (Table 24).

Survival data for disease free-survival (DFS) was available for 52 queens. Sixty-four percent (33/52) of the cats with mammary carcinoma showed disease recurrence at the end of the follow-up period (2 years and 2 months), with the majority showing locoregional recurrence (24/33, 72.7%) and the rest distant metastases (27.3%, 9/33). The mean DFS was 13.4 ±1.82 months (95% CI: 9.8-17 months). Considering the overall survival (OS), data was available in 58 animals, from which twenty-nine (50%) died, presenting a mean survival of 27.18 ±3.4 months (95% CI: 20.5-33.9 months).

4.2. Cats with mammary carcinoma show elevated sHER2 levels

Serum HER2 levels were measured both by ELISA and by dot blot assay. The circulating HER2 levels were calculated from the standard curve of known rHER2-ECD concentrations (Figure 21). The intra-assay and inter-assay coefficients of variation were 6.2% and 4.6%, respectively.

Box plots were used to illustrate the full data range of each group and to identify outliers. Cats having sHER2 levels that fall more than three standard deviations away from the mean were considered outliers and removed from further analysis: ELISA results presented two outliers in the healthy group and seven in the cancer group, that were removed; there were no outliers in the dot blot results.

Table 24. Clinicopathological features of 60 female cats with mammary carcinoma

Clinicopathological feature	Number of animals (%)	Clinicopathological feature	Number of animals (%)
Breed		**Size**	
Not determined	46 (76.7%)	<2 cm	24 (40.0%)
Siamese	9 (15.0%)	2-3 cm	24 (40.0%)
Persian	3 (5.0%)	>3 cm	12 (20.0%)
Norwegian Forest Cat	2 (3.3%)	**[a]HP classification**	
Spayed		Tubulopapillary carcinoma	36 (60.0%)
No	30 (50.0%)	Solid carcinoma	13 (21.7%)
Yes	29 (48.3%)	Cribriform carcinoma	6 (10.0%)
Unknown	1 (1.7%)	Mucinous carcinoma	5 (8.3%)
Contraceptives		**Malignancy grade**	
No	18 (30.0%)	I	3 (5.0%)
Yes	31 (51.7%)	II	14 (23.3%)
Unknown	11 (18.3%)	III	43 (71.7%)
Treatment		**Necrosis**	
None	1 (1.7%)	No	20 (33.3%)
Mastectomy	54 (90.0%)	Yes	40 (67.7%)
Mastectomy + Chemo	5 (8.3%)	**Lymphatic invasion**	
Multiple tumors		No	49 (81.7%)
No	22 (36.7%)	Yes	11 (18.3%)
Yes	38 (63.3%)	**Lymphocytic infiltration**	
Lymph node status		No	24 (40.0%)
Negative	32 (53.3%)	Yes	36 (60.0%)
Positive	23 (38.3%)	**Tumor ulceration**	
Unknown	5 (8.3%)	No	57 (95.0%)
Stage (TNM)		Yes	3 (5.0%)
I	13 (21.7%)	**Ki 67 index**	
II	9 (15.0%)	Low (< 14%)	24 (40.0%)
III	29 (48.3%)	High (≥ 14%)	35 (58.3%)
IV	9 (15.0%)	Unknown	1 (1.7%)
Localization		**PR status**	
M1	9 (15.0%)	Negative	28 (46.7%)
M2	12 (20.0%)	Positive	32 (53.3%)
M3	24 (40.0%)	**ER status**	
M4	14 (23.3%)	Negative	40 (33.3%)
Unknown	1 (1.7%)	Positive	20 (66.7%)
		***f*HER2 status**	
		Negative	38 (63.3%)
		Positive	22 (36.7%)

[a]HP classification, Histopathological classification

ELISA results revealed that cats from the cancer group showed significantly higher sHER2 levels (mean=22.3 ng/ml; range of values: 0-147.05 ng/ml) than healthy animals (mean=18.3 ng/ml; range of values: 0-104.2 ng/ml), with a significant p-value of 0.04 (Figure 22A). A more significant difference between the sHER2 levels of the two animal groups was found when dot-blot assay was performed (p=0.01), with the cancer group showing a mean value of sHER2 of 14 ng/ml (range of values: 0-75 ng/ml) and the healthy group a mean value of 4.5 ng/ml (range of values 0-15 ng/ml), as depicted in Figure 22B.

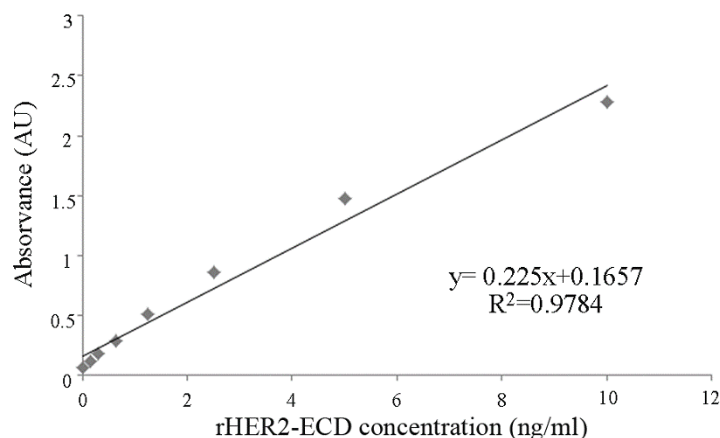

Figure 21. Standard curve for sHER2 measurements using a commercial ELISA kit.

The intra and inter-assay coefficients of variation were <10%.

4.3. Serum HER2 levels predict the tumor HER2 status

A significant difference was found between sHER2 levels of cats with HER2-negative mammary carcinoma (IHC-negative group) and sHER2 levels of cats diagnosed with mammary carcinoma overexpressing HER2 (IHC-positive group), both by ELISA (p=0.001, Figure 23A) and by Dot blot assay (p=0.03, Figure 23B).

Additionally, a strong correlation was found between sHER2 levels quantified by ELISA and tumor HER2 status (r=0.58, p<0.0001), coupled with a moderate association between sHER2 levels measured by Dot blot assay and tumor HER2 status (r= 0.26, p<0.1). Moreover, the Kappa coefficient showed a moderate agreement between ELISA and IHC results (k=0.48, p=0.002), and a fair agreement among Dot blot and IHC results (k=0.264, p=0.047). However, results obtained by ELISA and Dot blot assay only showed a fair agreement (k=0.27, p=0.048).

4.4. Serum HER2 molecules contain a portion of the intracellular receptor domain

In order to validate the measurement of sHER2 levels by the dot blot assay, the antigenic specificity of the anti-HER2 monoclonal antibody (clone SP3) was evaluated through western blot analysis. As expected, the SP3 antibody recognizes a protein band of ~185 kDa corresponding to predicted molecular weight of full-length HER2 in whole cell extracts of both human breast cancer cells (SKBR3, Figure 24A, line 1) and feline mammary tumor cells (FMCp, Figure 24A, lane 2), and cross-reacts with the recombinant human HER2-ECD (Figure 24A, lane 3, 84-90 kDa).

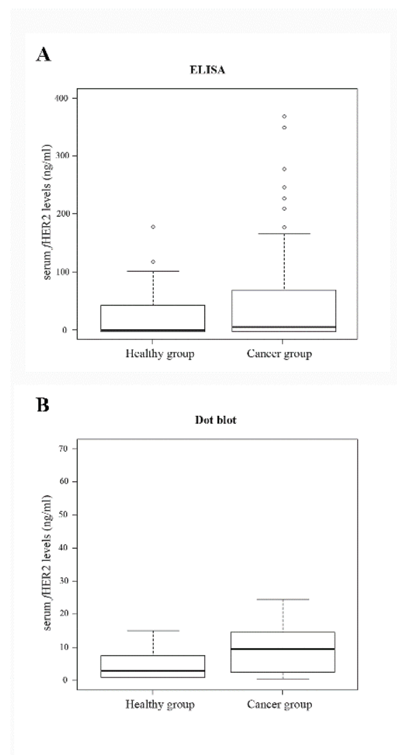

Figure 22. Box plot diagrams representing the sHER2 levels in control cats (healthy group) and in cats with mammary carcinoma (cancer group) determined by ELISA (A) and dot blot (B) assay.

Cats with mammary carcinomas had significantly higher mean of sHER2 levels than healthy cats both by ELISA (*p*=0.04) and dot blot (*p*=0.01). Outliers are indicated by open circles.

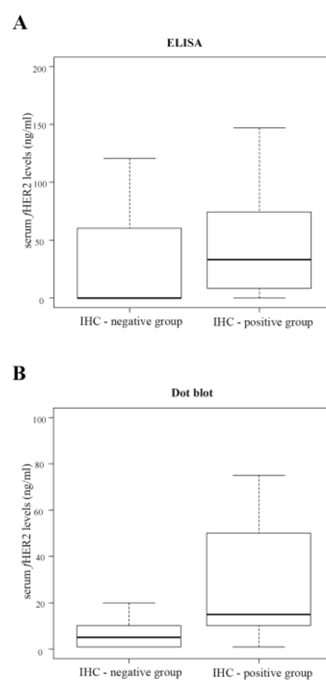

Figure 23. Box plot diagrams showing that tumor HER2 status correlates with sHER2 levels as assessed by both ELISA (A) and Dot-blot assay (B). (legend continues next page)

Figure 23. (continuation of the legend) (A) A significant difference was found (*p*=0.001) between the sHER2 levels of cats with MC overexpressing HER2 (IHC-positive group: mean 42.6 ng/ml [range of values 0-147.05 ng/ml]) and the sHER2 levels of cats with HER2-negative MC (IHC-negative group: mean 8.8 ng/ml [range of values 0-73.89 ng/ml]), using ELISA (*p*=0.001). **(B)** The Dot blot assay also showed a significant difference between the sHER2 levels measured in these two studied groups, with a *p*-value of 0.03 (IHC-positive group: mean 24.4 ng/ml [range of values 0-75 ng/ml]; IHC-negative group: mean 8 ng/ml [range of values 0-20 ng/ml]).

Further western blot analysis revealed that the anti-HER2 monoclonal antibody detected several protein bands with masses ranging between 120 and 140 kDa in the serum samples from cats diagnosed with MC overexpressing HER2 (Figure 24A, lanes 4 and 5), suggesting that proteolytic cleavage of HER2 occurs at the intracellular domain (ICD), leading to the production of truncated HER2 soluble forms which are quantifiable by the Dot blot assay (Figure 24B). As mentioned above, cats diagnosed with HER2-overexpressing MC (scored as 2+ and 3+), showed significantly (*p*= 0.03) higher sHER2 levels (Figure 24B, lines 5 and 6) than cats with HER2-negative mammary carcinomas (Figure 24B, lines 3 and 4) or healthy cats (Figure 24B, line 2).

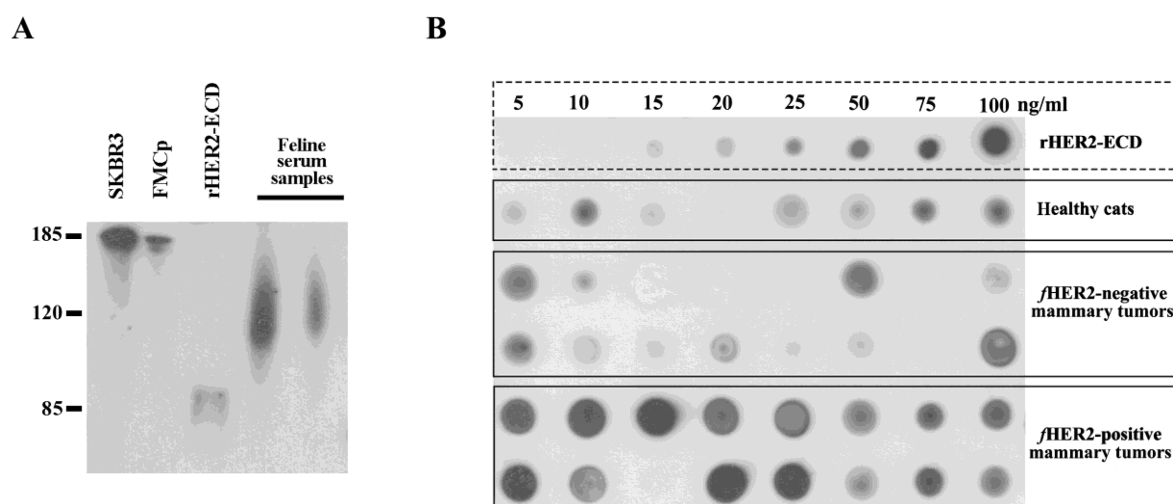

Figure 24. Soluble truncated HER2 forms carry a portion of the ICD and are quantifiable by Dot blot assay. (A)

The anti-HER2 SP3 antibody specifically recognizes a protein band of about 185 kDa in human and feline whole cell extracts (SKBR3 and FMCp, lanes 1 and 2), the purified rHER2-ECD (lane 3, ~75 kDa), and a protein with a molecular weight that ranges between 100 and 140 kDa in cat serum samples (lanes 4 and 5). **(B)** A representative image of an immunoblot performed with serum samples collected from healthy cats (line 2) and cats with mammary carcinoma (lines 3-6). sHER2 levels were semi-quantified by comparing the intensity of each serum dot with the intensities of the dots obtained from rHER2-ECD dilutions (line 1).

4.5. sHER2 levels ≥10 ng/ml are the optimal cut-off value to diagnose HER2-overexpressing FMC

To determine the best cut-off value, the sensitivity and the specificity of both ELISA and Dot blot assay were determined using different cut-off points to classify cats according to their sHER2 levels. ROC curve analysis revealed that the best cut-off was ≥10 ng/ml to discriminate cats with mammary carcinomas overexpressing HER2 (Table 25). By using this threshold value, the sensitivity and the specificity of ELISA were 69% and 67%, respectively (Figure 25A, [AUC=0.70, 95% CI=0.55-0.85]), while the Dot blot assay revealed a sensitivity of 53% and a specificity of 78% (Figure 25B, [AUC, 0.73; 95% CI=0.58-0.88]).

6 out of 20 healthy cats (30%) had elevated sHER2 levels (≥10 ng/ml) when ELISA was used and 5/20 (25%) with the Dot blot assay. This is in accordance with studies in humans, where false-positive rates can reach up to 20% (Lam *et al.*, 2012).

Table 25. Serum HER2 levels in cats with mammary carcinoma

Immunoassay	Cats (n, %)	Mean and range values (ng/ml)
ELISA (n=40)		
Low levels (< 10 ng/ml)	24 (60.0%)	0.35 (0.00-8.38)
High levels (≥ 10 ng/ml)	16 (40.0%)	55.17 (13.75-147.05)
Dot blot (n=47)		
Low levels (< 10 ng/ml)	20 (42.6%)	2 (0-5)
High levels (≥ 10 ng/ml)	27 (57.4%)	22.78 (10-75)

4.6. Elevated sHER2 levels are associated with a less aggressive tumor phenotype

ELISA results revealed that cats with sHER2 levels ≥10 ng/ml were significantly associated with the early stage of the disease (stage I, p=0.006; Odds Ratio [OR]=17.9; 95% CI: 1.633-984.42), absence of tumor necrosis (p=0.0024; OR=9.28; 95% CI: 1.79-60.61) and lower Ki-67 scores (<14%, p=0.007; OR=7.73; 95% CI: 1.57-47.54). In parallel, the Dot blot results also correlate with high sHER2 levels (≥10 ng/ml) with cats that have mammary carcinomas with low Ki-67 index (p=0.016; OR=5.24; 95% CI: 1.23-27.79). Supporting the above results, significant correlations were found between the HER2-negative tissue status and the presence of tumor necrosis (p=0.0012; OR=7.16; 95% CI: 1.91-30.5), moderate malignancy grade (p=0.049; OR=3.4; 95% CI: 0.8-14.6), low Ki-67 values (p=0.0022; OR=6.42; 95% CI: 1.81-25.5) and ER positivity (p=0.049; OR=3.15; 95% CI: 0.91-11.45).

5. Discussion

Acquired growth signals autonomy was the first identified hallmark for cancer, mainly due to the discovery of oncogenes that modulate the cell cycle leading to an unregulated proliferation (Hanahan & Weinberg, 2000). In humans, the overexpression of the proto-oncogene HER2 is responsible for 15-30% of all breast cancers and is associated with a poor outcome, although specific therapy with anti-HER2 antibodies and tyrosine kinase inhibitors has been shown to

prolong survival (Stebbing *et al.*, 2000). Recently, several studies have shown that HER2 overexpression occurs frequently in FMC (33-59.6%), although HER2 gene amplification was undetected (Soares *et al.*, 2013a). In this study, 36.7% (22/60) of the selected population had HER2-overexpressing mammary carcinomas, corroborating the previous published data (De Maria *et al.*, 2005; Millanta *et al.*, 2005a; Ordás *et al.*, 2007; Soares *et al.*, 2013a).

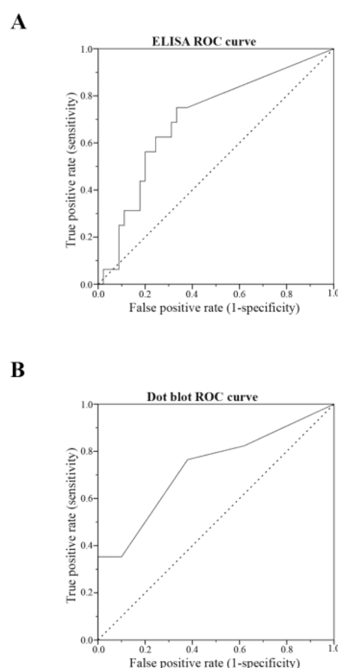

Figure 25. Receiver-operating characteristic (ROC) curve for sHER2 levels for ELISA (A) and Dot blot assay (B).

The optimal sHER2 cut-off value (10 ng/ml) was chosen to maximize the sum of the sensitivity and specificity on the Youden index (sensivity+specifity-1). The AUC was 0.70 (95% CI: 0.55-0.85) for ELISA **(A)** and 0.73 (95% CI: 0.58-0.88) for Dot blot assay **(B)**, demonstrating that the sHER2 levels are a good biomarker to differentiate cats with MC overexpressing HER2 from cats with MC non-expressing HER2 or healthy cats. When the cut-off value of 10 ng/ml was set, the sensibility was 69% and the specificity was 67% for ELISA, whilst for Dot blot assay the sensibility and the specificity were 53% and 78%, respectively.

To the best of our knowledge, this is the first work to detect and quantify the serum HER2 levels in cats, using two immunoassays: the ELISA which has already been approved to measure sHER2 levels in women with metastatic breast cancer, and the Dot blot assay, a less expensive technique (Tan *et al.*, 2011; Moelans *et al.*, 2011). Our results show that sHER2 levels in cats with HER2 overexpressing mammary carcinomas are significantly higher than in cats with HER2-negative mammary carcinomas (p=0.001) or healthy animals (p=0.04). As also reported for breast cancer patients, a false-positive rate of 25-30% was found in healthy cats, when either ELISA or Dot blot assays was performed (Kong *et al.*, 2012; Lam *et al.*, 2012; Tsé *et al.*, 2012).

Using ROC analysis the best cut-off value to identify cats with mammary carcinomas overexpressing *f*HER2 was 10 ng/ml in both techniques. Interestingly, this threshold is the one also used in woman with breast cancer (Carney *et al.*, 2003; Lam *et al.*, 2012). Comparing the two assays, ELISA was more sensitive in detecting cats with elevated sHER2 levels than Dot blot assay (69% versus 53%) but less specific (67% versus 78%).

Moreover, a moderate correlation was found between the sHER2 levels and the tissue HER2 status when ELISA was employed (r=0.58, p<0.0001), revealing that sHER2 levels predict the HER2 status of the primary mammary tumor. However, the correlation with the Dot blot assay was low (r=0.3). ELISA studies in humans showed contradictory results: some did not find any correlation between tissue and serum HER2 levels (Kong *et al.*, 2006a), whereas others reported a poor or a moderate/strong correlation (Kong *et al.*, 2006c; Ludovini *et al.*, 2008; Kong *et al.*, 2012; Lam *et al.*, 2012). Regarding the dot blot technique, there is only a study that correlate the tissue and serum HER2 levels, and presents a similar result with this study (r=0.3) (Tan *et al.*, 2011).

Results from the western blot analysis demonstrated that the soluble HER2 fragments also contain a portion of the intracellular domain, since a protein band between 100 and 135 kDa was detected. This result was additionally supported by the use of two anti-HER2 antibodies that recognize the intracellular domain of HER2 (4B5 and A0485) in the dot blot assay, where a similar signal intensity to the SP3 antibody was obtained.

Further statistical analysis revealed that cats with MC showing HER2 overexpression or high sHER2 levels were associated with features usually associated with a better outcome (low Ki-67 index, absence of tumor necrosis, ER-positive status and lower disease stages). Previous studies in cats did not show significant associations (Winston *et al.*, 2005; Millanta *et al.*, 2005a; Rasotto *et al.*, 2011). Although HER2-positive tumors are associated with larger lesions, lymph node infiltration, high grade of malignancy, absence of hormone receptor expression and proliferation index in breast patients (Ménard *et al.*, 2000; Ludovini *et al.*, 2008; Kong *et al.*, 2012), our results could be due to the fact that most of HER2-overexpressing FMC also presented a positive ER and/or PR status, which can be classified as luminal B and not as HER2-positive immunophenotype. Indeed, luminal B breast tumors in humans showed a less aggressive behavior than HER2-positive tumors (Goldhirsch *et al.*, 2013).

In summary, this study revealed that cats with mammary HER2-overexpressing carcinomas showed elevated sHER2 levels, which can be detected and quantified by ELISA and dot blot assay. Even though more studies are needed to clarify the role of HER2 in the pathogenesis of FMC, our results showed that sHER2 levels measurement are useful to diagnose FMC overexpressing HER2, and probably, to predict the therapeutic response to anti-HER2 agents (antibodies or kinase inhibitors), as in humans, where a rapid decrease of the sHER2-ECD levels after treatment is indicative of a good therapeutic response (Fornier *et al.* 2005; Kong *et al.*, 2006b; Kong *et al.*, 2012).

This study has shown for the first time that the serum *f*HER2-ECD levels can be classified using two different techniques, the ELISA and the Dot blot assay. Thus, demonstrating that *f*HER2 protein can release its extracellular domain into the extracellular space, an event that is apparently similar to the one described in humans. ELISA appears to be the best technique, giving a higher correlation with the tissue level and although less specific than Dot blot assay, could be a supplement to the IHC with possible applications in Veterinary Medicine. High serum *f*HER2-ECD levels are present in 40% of the feline patients (using the ELISA assay) and overexpression of *f*HER2 protein was observed in 36.7% of the population (with IHC).

Surprisingly, the *f*HER2 protein in serum and in tissue was associated with less aggressive features, contradicting what is described for humans. These findings, together with the non-amplification of the *f*HER2 gene (Soares *et al.*, 2013a), reinforce that more studies are needed in order to clarify the biological role of this protein.

Finally, the high prevalence of this protein in FMC could be important for the study of specific target therapies directed against HER2 protein, both in a veterinary clinical perspective, improving the therapeutic options of these animals and also in a comparative perspective, using the cat as a model for therapeutic trials.

GENERAL DISCUSSION

CONCLUSION

FUTURE PERSPECTIVES

GENERAL DISCUSSION

Studies on feline mammary carcinomas had two main objectives: to increase knowledge on this veterinary oncology field and to understand if the cat can be used as a suitable model in breast cancer research.

Veterinary Comparative Oncology is a recent field that integrates the naturally occurring cancers of companion animals into more general studies of cancer biology and therapy (Paoloni & Khanna, 2008). Nowadays, this discipline gained significant importance as it provides novel opportunities for the advance of diagnosis, treatment and prevention in veterinary and human cancer patients.

Cat in parallel with dog, are a popular companion animals in Europe and in the USA (Downes *et al.*, 2009; Murray *et al.*, 2010; Murray *et al.*, 2015). In 2011, the estimated UK cat population was 10 114 764 cats, which is quite similar to the estimated dog population – 11 599 824 dogs (Murray *et al.*, 2015). In the USA, data from 2012 indicates that there are approximately 74 059 000 cats and the estimated number of dogs is 69 926 000 (American Veterinary Medicine Association [AVMA], 2012). In addition, cancer in companion animals is the major cause of death and morbidity, and at least 4 million cats (and 4 million dogs) develop this disease every year. Therefore, this scenario fully justifies the critical importance of this disease in veterinary practice (Withrow *et al.*, 2013). Additionally, the high incidence also provides a large enough sample that ensures the achievement of valid results in cancer studies.

Dogs are already widely used as animal models in cancer research studies, whilst cat's studies are more scarce (Zappulli *et al.*, 2005; Paoloni & Khanna, 2008; Pinho *et al.*, 2012). The thesis herein presented aimed to increase knowledge about FMC and to understand whether the cat can be a suitable model to study breast cancer disease. Considering the available literature, most of the feline mammary tumors are malignant and HR-negative (de las Mulas *et al.*, 2000; de las Mulas *et al.*, 2002; Millanta *et al.*, 2005b; Millanta *et al.*, 2006a; Burrai *et al.*, 2010; Sorenmo *et al.*, 2013), supporting that cat species are potential models for HR-negative breast cancers. In addition, those are also compared to the most severe subtypes of human breast cancer, with less effective therapies and poor outcomes.

Considering this, the focus of this thesis was the evaluation of *f*HER2 expression in FMC. The sequence homology of HER2 gene between Dog and Human is extreme similar (80%[1]) as between Cat and Human (79%[2]) and the gene sequences are available at Genebank (GenBank NC_006591.3[3]; NC_018736.2[4]; NC_000017.11[5]). Moreover, Cat HER2 and TOP2A genes are located in the same chromosome, in the long arm of chromosome E1, as described for Human (in chromosome 17) and Dog (chromosome 9) genes.

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[1]NCBI Blast: http://blast.ncbi.nlm.nih.gov/Blast.cgi (31.01.2016)
[2]NCBI Blast: http://blast.ncbi.nlm.nih.gov/Blast.cgi (31.01.2016)
[3]GenBank: http://www.ncbi.nlm.nih.gov/gene/403883 (31.01.2016).
[4]GenBank: http://www.ncbi.nlm.nih.gov/gene/751824 (31.01.2016).
[5]GenBank: http://www.ncbi.nlm.nih.gov/gene/2064 (31.01.2016).

At protein level, there is a high homology between feline and human HER2 sequences (93.7%), quite similar to the homology found between dog and human HER2 sequences (92.9%)[6].
Interestingly, cat presents specific characteristics concerning HER2 positive tumors in humans, that best support studies in this species when compared to dog. As described before, most of the feline mammary tumors are malignant (unlike dogs, where benign tumors reach up to 50% of all mammary tumors). Usually, these are hormone independent and present a moderate response to chemotherapy protocols, especially to doxorubicin, like described for human patients with HER2-positive mammary tumors (Novosad *et al.*, 2006; McNeill *et al.*, 2009; Euler, 2011; Goldhirsch *et al.*, 2013; Sorenmo *et al.*, 2013; Salas *et al.*, 2015).

As a consequence of the scarce knowledge, the first two chapters of this thesis intended to provide methodologies to assessed the biomarkers in study. Herein, it was demonstrated that a considerable population of mammary tumors (about 30%) present *f*HER2 overexpression, as described in previous studies (De Maria *et al.*, 2005; Ordás *et al.*, 2007), and in similarity to what is described for women (Wolff *et al.*, 2013).
Nonetheless, studies performed by our group confirmed the absence of *f*HER2 gene amplification in cats. In fact, we recently performed FISH assays in 19 FMC with *f*HER2 overexpression where gene amplification could not be detected (Figure 26).

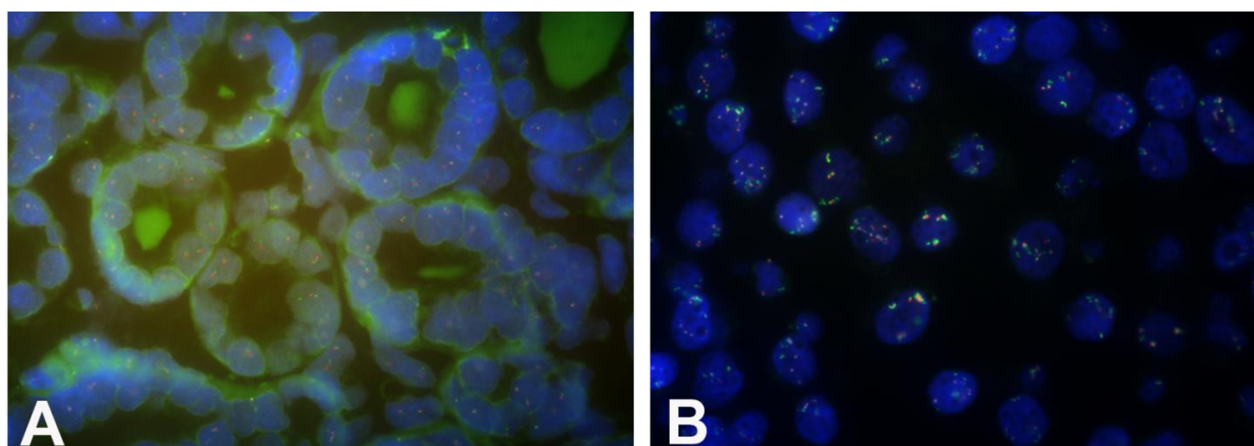

Figure 26. Evaluation of *f*HER2 (green signal) and *f*TOP2A (red signal) gene expression by FISH.

A. FMC with *f*HER2 overexpression and gene amplification absence. **B.** Human mammary carcinoma cell line (SKBR3) showing HER2 and TOP2A gene amplification.

We also analyze the copy numbers of *f*HER2 gene in FMC and normal mammary tissue samples of 25 queens, using qPCR. Notably, only two samples presented an increase of *f*HER2 gene copy number (Figure 27), which could be due to gene amplification or chromosome polysomy. Overall, these results strengthen the hypothesis that *f*HER2 overexpression in FMC is caused by other mechanism rather than gene amplification, such as transcription misregulation or gene mutations.

[6] Uniprot: http://www.uniprot.org/blast/uniprot/B2016013175SEOLU5H9 (31.01.2016)

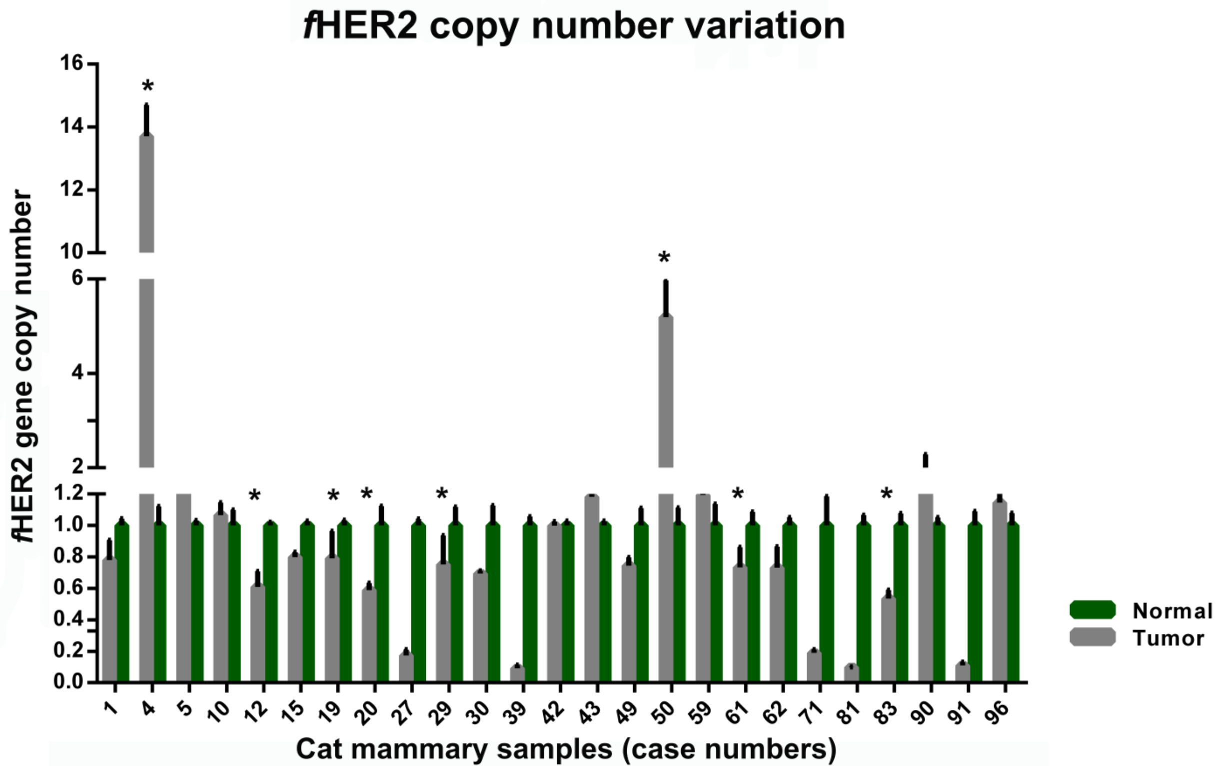

Figure 27. *f*HER2 gene copy number in mammary carcinomas and normal mammary feline tissue samples.

The graphic illustrates the copy number variation of *f*HER2 gene in comparison to normal mammary gland tissue of the same animal. The * refers to the cases presenting *f*HER2 overexpression using IHC.

Despite no literature is available regarding gene deregulation in feline or canine mammary carcinomas, several studies already evaluated this mechanism in human breast cancer. Histone acetylation and DNA methylation are epigenetics events being critical regulators of chromatin structure, which are key events of gene transcription. Studies indicated that epigenetic changes that led to gene deregulation could be implicated in cancer development. In fact, changes in chromatic structure can led to inappropriate gene silencing, such as inhibition of tumor supressors genes, or to inappropriate gene activation, such as oncogenes transcription (Mielnicki *et al.*, 2001; Subramaniam *et al.*, 2009).

Regarding the HER2-positive tumors, Lindqvist and colleagues (2014) demonstrated an extensive hypermethylation of CpG islands in these tumors. This hypermethylation modulates transcription factors, especially the homeobox genes and other members of HER2 signalling pathways. Indeed, this research demonstrated different methylation status of PI3K/AKT-related genes, namely HER2 and AKT, which became hypomethylated and thus more activate.

The homeobox genes are known to be transcriptional regulatory proteins, encoding for homeoproteins and controling embryogenesis. Also the deregulation of this homeoproteins has been associated with cancer (Abate-Shen, 2002; Shah & Sukumar, 2010).

Another mechanism for gene deregulation is the modified transcription of microRNAs. While histone acetylation and DNA methylation are determinant in gene expression, microRNAs are small endogenous non-coding RNAs (19-25 nucleotides long) and are fundamental in post-transcriptional regulation (Melo & Esteller, 2011; Sandhu *et al.*, 2014). Therefore, and similarly to others cancer types, human breast cancer was already associated to altered miR expression. miRs have been implicated in the regulation of numerous biological processes such as cellular proliferation, differentiation, development and apoptosis (Sandhu *et al.*, 2014). In breast cancer, altered expression of different miRs have already been associated with tumor stage, HRs expression, proliferation index, vascular invasion, epithelial to mesenchymal transition, metastasis and neovascularization (Iorio *et al.*, 2005; Sandhu *et al.*, 2014).

In HER2 positive subtype tumors case, a recent study demonstrated an altered expression of some miRs, although their involvement in HER2 pathways is still not well understood (Tashkandi *et al.*, 2015).

Additionally, alternative splicing could also be responsible for gene deregulation, once it can produce truncated proteins with oncogenic activity, like Δ16HER2 (Jackson *et al.*, 2013; Wang *et al.*, 2013a; Inoue & Fry, 2015; Naftelberg *et al.*, 2015). In FMC, Santos *et al.* (2013) found a positive correlation between *f*HER2 RNA copies and *f*HER2 protein expression levels, when ICD was assessed. However, the same study demonstrated an absence of correlation between *f*HER2 RNA quantification and *f*HER2 protein levels when ECD was studied, whereby different alternative transcripts can be involved in FMC with overexpression of *f*HER2, as described in humans.

Finally, the presence of mutations is another potential explanation for *f*HER2 overexpression without gene amplification in FMC, and single point mutations have already been identified in feline carcinomas (Santos *et al.*, 2013).

In chapter II, an optimal cut-off value for Ki-67 index in FMC was established. This is a reference biomarker used by the St Gallen International Expert Consensus panel. Besides the importance of this biomarker to distinguish between luminal A and luminal B/HER2-negative tumors, our research revealed that Ki-67 index can also be considered has a prognostic factor in FMC (Soares et al., 2016b). Thus, its determination could improve the clinical management of cats, once high Ki-67 index levels (≥14%) were associated with lower OS and aggressive clinicopathological features.

Surprisingly, in this study, *f*HER2 overexpression was associated with low Ki-67 indexes (<14%), which raises questions whether the oncobiological role of *f*HER2 is maintained among cat and human. The scientific literature is inconsistent, with one study correlating *f*HER2 overexpression with lower OS times (Millanta *et al.*, 2005a) while another correlates lower HER2 RNA levels with poor outcomes (Santos *et al.*, 2013).

In chapters III and IV, FMC were characterized by determining the molecular subtypes of both primary and metastatic lesions.

In chapter III, the panel of biomarkers recommended by St Gallen International Expert Consensus Panel was used to divide FMC into different molecular subtypes. This approach provides new insights in feline and comparative oncology. This methodology enabled the split into the six subtypes and the confirmation of several clinicopathological features that are conserved between cat and human. Moreover, significant differences between the luminal B/HER2-positive and HER2-positive subtypes were observed, possibly justifying the described data in chapter II and V.

In human breast cancer, luminal B/HER2-positive is less endocrine sensitive, more aggressive and presents a worse prognosis when compared to luminal A subtype (Creighton. 2012; Goldhirsch *et al.*, 2013; Inic *et al.*, 2014). However, in comparison to HER2-positive non-luminal subtype, the luminal B/HER2-positive subtype presents a better prognosis (Sørlie *et al.*, 2003; Staaf *et al.*, 2010). The study presented in chapter III corroborates these facts in FMC (Soares *et al.*, 2016a). Considering the luminal B/HER2-positive and the HER2-positive subtypes, it was possible to identify distinct characteristics among them: HER2-positive (non-luminal) tumors were associated with larger and undifferentiated neoplasias (Grade III), presence of necrotic areas, higher Ki-67 index levels and lower OS and DFS, when compared to the luminal B/HER2-positive subtype.

Therefore, these differences could explain the apparent relation of *f*HER2 overexpression with more benign features (Chapter II and V), once most *f*HER2 overexpressing tumors are included in the luminal B/HER2-positive subtype. In addition, a relevant percentage of *f*HER2 negative tumors belong to the triple negative subtype (about 30%) and, consequently, an important proportion of *f*HER2 negative tumors are associated with aggressive features.

Regarding comparative oncology, cats present distinct frequencies of molecular subtypes when compared to humans. In humans, luminal A subtype is the most common, whereas in cat the most common tumors are luminal B and TN subtypes. Interestingly, these subtypes also correspond to the ones with less effective and specific therapies (Prat & Perou, 2011; Creighton, 2012). Thus, FMC could represent a suitable model to study new therapeutic drugs targeting such subtypes.

In chapter IV, the goal was to verify and characterize the heterogeneity of metastasis, by individually evaluating the biomarkers and the molecular subtypes. In Feline Oncology, data about mammary carcinoma disease progression is outdated and scarce (Hayden & Nielsen, 1971; Weijer & Hart, 1983; Hahn *et al.*, 1994). Recently, two studies (Brunetti *et al.*, 2013; Beha *et al.*, 2014) evaluated the molecular profile of FMC, also in regional and distant metastasis, but the small number of animals (n=3) avoided concordance studies between primary and distant lesions (Beha *et al.*, 2014).

Indeed, the metastatic progression remains the major challenge in oncologic patients and the molecular characterization of the relapse lesions is essential for the optimal management of these patients (Chan *et al.*, 2012). For that, both the receptor expression and the molecular profile were determined in primary human breast cancer and in corresponding metastatic lesions in several studies. Results published indicate that protein expression is not maintained during the progression

of the disease, even though discordant results were found between primary and relapse lesions (Simmons *et al.*, 2009; Aitken *et al.*, 2010; Jabbour *et al.*, 2012; Yang *et al.*, 2014).

About FMC, we demonstrated that the majority of relapse lesions do not preserve the immunophenotype profile detected in primary tumors. Moreover, it was noted that DM usually lose HRs expression and show a higher incidence of TN basal-like subtype, which is the molecular profile with poorer outcome.

Considering *f*HER2 expression, low concordance rates were found between PT and relapse lesions. In fact, more than half of animals with *f*HER2-positive mammary tumors presented *f*HER2-negative metastatic lesions (54.5%).

In sum, both in human and in feline mammary carcinomas, a tendency of ER, PR and HER2 expression loss is present. The reason for this loss is still not well understood, but we hypothesize that it should be important to allow tumor cells to acquire metastatic capacities and to survive in different tissue environments. In fact, the metastatic process is complex and is usually represented by five steps as illustrated in Figure 28 and briefly described below (Fidler, 2003; Alizadeh *et al.*, 2014):

1. Dissociation: when tumor cells detaches from the primary tumor.
2. Invasion: the detached tumor cells are able to infiltrate and migrate into the surrounding stroma, including through the basement membrane of the endothelium vessels. This step is a landmark event, where a locally growing tumor acquires ability to metastize, becoming a systemic disease.
3. Intravasation: is the process whereby the tumor cells enter in the vascular or lymphatic circulation. In circulating vessels, they have to be able to survive to anoikis (Argyle & Khanna, 2013; Alizadeh *et al.*, 2014). If these tumor cells survived in the circulation, they become trapped in the capillary vessels of distant organs, through adhesion to endothelial cells or to subendothelial basement membrane (Fidler, 2003).
4. Extravasation: the tumor cells actively leave the vasculature towards distant and different organs. There, they have to survive in a new hostile microenvironment.
5. Dormancy: After reaching their target organ, metastatic tumor cells remain dormant for a period of time (Argyle & Khanna, 2013).

In the **dissociation process**, the individual tumor cell loses its ability to adhere to other tumor cells. The loss of cell-to-cell adhesion is essential for the tumor cell disaggregation and the promotion of the initial dissemination (Alizadeh *et al.*, 2014). The cell-to-cell adhesion molecules are glycoproteins, where cadherins are included. Thus, several studies associate the downregulation or loss of cadherins to an increased metastatic potential (reviewed in Beavon, 2000).

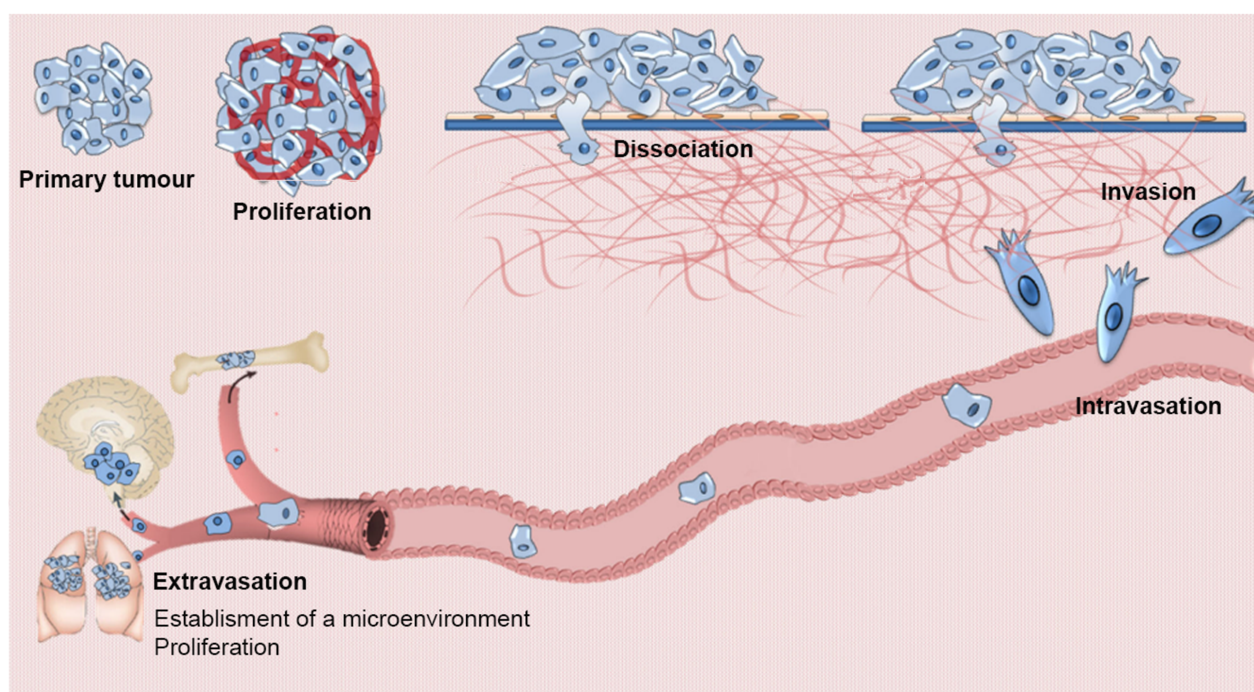

Figure 28. The metastatic cascade.

The figure represents a set of steps that tumor cells must undergoes to be able to originate metastasis. The switch from normal tissue to primary cancer lesion is the beginning of this process, where complex alterations occurs to grant primary tumor development. **Dissociation** and **Invasion** allow cancer cells to locally invade the surrounding stroma and to migrate through the basement membrane of the endothelium. Then, the tumor cells enter into blood and/or lymphatic vessels (**Intravasation**). After resisting anoikisis tumor cells became trapped in the capillary vessels and **Extravasation** occurs. Finally, the invaded tumor cells can remain silent for uncertain periods of time (**Dormancy**). When these cells start to proliferate the metastatic process is complete and after that they can again invade blood vessels, enter into bloodstream and originate more metastasis. *Adapted from* Alizadeh *et al.* (2014).

In the **invasion step** cells acquire the capacity to migrate while several signalling pathways are involved. Epithelial tumor cells have to lose their characteristics such as cell-cell adhesive structures, to change their polarities and to pass through extensive reorganization of their cytoskeletal structure (Al Saleh *et al.*, 2011). These transformations occur by several changes in gene expression, which are responsible for a decrease of the epithelial markers and an increase of mesenchymal markers, responsible for the called epithelial to mesenchymal transition (EMT). Among the different factors that regulate EMT, TWIST is one of the most important (Zhang *et al.*, 2014; Zhang *et al.*, 2015). Recently, studies demonstrated that triple negative breast tumors (ER-, PR- and HER2-) are associated with an enrichment of stem cell-like, EMT markers and TWIST activation (Ye *et al.*, 2008; Al Saleh *et al.*, 2011; van der Horst *et al.*, 2012; Bouris *et al.*, 2015; Zhang *et al.*, 2015).

Taking all this in consideration, it was hypothesized that the loss of the HR expression in the metastatic lesions, coupled with an increased percentage of TN basal-like subtype lesions, could be important to allow tumor cells to suffer the transformations of the metastatic cascade, such as

the EMT. In light of the above data, maybe the loss of the HR expression in FMC is associated to an increased expression of TWIST and to a downregulation of the E-cadherins.
Unfortunately, studies concerning EMT in FMC are not available. Nevertheless, Peñafield-Verdu *et al.* (2012) found a correlation between reduced E-cadherin and β-catenin expression in FMC and the presence of regional metastasis. Further, Baptista *et al.* (2012) showed that FMC had significantly lower TWIST mRNA levels than benign mammary lesions.

In summary, chapters III and IV revealed that FMC can be classified in six distinct molecular subtypes. This classification has proven to have prognostic value in cat and open new perspectives to develop specific therapies. Thus, we have already started to perform *in vitro* assays, using TUNEL assay, testing the anti-tumor activity of a commercial antibody anti-HER2 (trastuzumab) in feline tumor cells (Soares *et al.*, 2012). Preliminary results uncovered an increased cellular mortality rate in cells related with this immunoagent.
The significant differences in HRs status and in molecular profiles between primary and relapse lesions require a full evaluation of all the lesions (primary and metastatic), especially if, in future, a targeted therapy becomes available to treat FMC. This represents a relevant drawback both for human and veterinary medicines, once metastatic lesions are sometimes inaccessible to perform a biopsy, leading to a relevant increment of costs in these patients management (Kong *et al.*, 2012). Thus, the development of non-invasive techniques that allows the continuous follow-up of this disease after the invasive surgery represents a major progress in the clinical management. One of the most promising strategies to better evaluate clinical status is the identification and quantification of tumor biomarkers in the serum, like sHER2 (Esteva *et al.*, 2005; Ludovini *et al.*, 2008; Kong *et al.*, 2012).
Thus, the focus on sHER2 that led to the study presented in chapter V, emerges from the high prevalence of FMC-HER2+ cases (about 30%), the low OS and low DFS observed in cats with HER2-positive (non-luminal) subtype and the high discordance rate of receptor expressions among the different lesions.

In chapter V, the *f*HER2 fraction that is shed from the surface of the cell and can be detected in the blood circulation, as occurs in humans, was investigated. In addition, the study also intended to provide new diagnostic tools to identify cats with *f*HER2 positive mammary carcinomas.
The results obtained show that a soluble fragment of *f*HER2 protein could be detected in blood circulation, probably from *f*HER2 shedding, as described in human breast cancer patients. The correlation between *f*HER2 tissue status (IHC) and serum *f*HER2 levels (ELISA) indicates that probably *f*HER2 is also subjected to a shedding process. Interestingly, the soluble *f*HER2 fragment detected includes a portion of the intracellular domain, a feature different from what is described in human patients. In women it was established that HER2 shedding is caused by proteins belonging to ADAM and MMP families (Sanderson *et al.*, 2006; Tsé *et al.*, 2012), while in cats the shedding

mechanism is still unclear. However, one study that compared the normal and the tumor tissue levels of MMP-9 in cats concluded that mammary carcinomas present higher levels and higher enzymatic activity of MMP-9 (Akkoc *et al.*, 2012).
Finally, our group recently measures the MMP-2 levels in FMC and studied their correlation with several characteristics, including the *f*HER2 tissue levels (André, 2015). Despite the results obtained could not prove a statistical significance (p=0.063), most of the tumors with *f*HER2 overexpression showed high MMP-2 levels, whereby its possible role in the *f*HER2 shedding should be further investigated.

CONCLUSIONS

The studies herein included provide new clinical, diagnostic and prognostic tools for FMC.
It was demonstrated that FMC can be divided according to their immunophenotype profile, as described for humans. Similarly, luminal A subtype presents a better prognosis when compared to the other subtypes, and both HER2-positive (non-luminal) and triple negative basal-like subtypes were associated with the lowest OS and DFS times. This knowledge provides relevant information to support different therapeutic protocols in cats with mammary tumors.
Further, a loss in the HRs expression, in the metastatic lesions, was found. Therefore, we hypothesized that this event could be related with the trans-differentiation from an epithelial to a mesenchymal phenotype (EMT), which is associated with the dissociation and invasion steps of the metastatic process.
Studies conducted also indicate that *f*HER2 overexpression is not associated with gene amplification, unlike in humans. In order to understand the mechanism underlying this overexpression, more studies are required. Finally, we demonstrated that *f*HER2 could be measured in blood samples, which could represent a novel diagnostic tool, useful to diagnose *f*HER2-positive tumors and to follow-up the disease in cats.
Besides the clinical applications, the studies presented are also important for comparative oncology, once FMC presents similarities with the human breast cancer. Additionally, luminal B and triple negative subtypes are the most prevalent subtypes in cats and, in humans, are the ones that do not have specific treatment. Consequently, we considered that this species could be a suitable model to study human luminal B and triple negative subtypes.

FUTURE PERSPECTIVES

Assuming the aggressiveness of FMC, the absence of effective treatments and the potential applications in translational medicine, further investigation on molecular characterisation of FMC are needed.

Results presented in this thesis indicate that FMC is a very heterogeneous disease. Despite a relevant percentage of FMC overexpresses *f*HER2, the mechanism underlying this overexpression is still not understood. Thus, it would be relevant to investigate *f*HER2 gene regulation, possible genomic mutations, *f*HER2 mRNA levels, and the proteins involved in the *f*HER2 activated signalling pathways.

Based on our results, there is a high prevalence of discordant protein expression between primary and metastatic lesions. Thus, it would be relevant to understand the marked loss of receptor expression in the last, once again through the study of the signalling pathways that are activated or supressed with the overexpression or downregulation of these receptors in the different primary and metastatic lesions. Also, these results require the development of new diagnostic tools that are able to evaluate the metastatic disease at real-time, with non-invasive techniques and ideally, sensible to identified alterations even before the metastatic lesion becomes macroscopically visible. In this perspective, we considered that more studies should be performed, in order to optimize the detection of *f*HER2 in serum samples, to evaluate if it could be used to follow-up the progression of the disease in cats and to monitor the treatment efficacy.

Finally, the high prevalence of luminal B and triple negative subtypes in FMC should be addressed carefully, in order to identify diagnostic and therapeutic targets that will allow the use of these animals as a model for therapeutic trials in cancer research.

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ANNEX I – Feline HER2 Protein Expression Levels and Gene Status in Feline Mammary Carcinoma: Optimization of Immunohistochemistry (IHC) and In Situ Hybridization (ISH) Techniques

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Feline HER2 Protein Expression Levels and Gene Status in Feline Mammary Carcinoma: Optimization of Immunohistochemistry (IHC) and *In Situ* Hybridization (ISH) Techniques

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Abstract: Human epidermal growth factor receptor (HER2) is a tumor biomarker that when overexpressed and/or amplified is associated with a poor prognosis for women with breast cancer. This specific tumor subtype is eligible for a specific immunotherapy that increases survival period. However, in feline oncology, only a few studies have been performed on molecular characterization of feline (*f*HER2) in feline mammary carcinoma (FMC), and the available data are inconsistent. In this study, *f*HER2 protein levels and gene status in FMC were evaluated by immunohistochemistry and *in situ* hybridization. After being optimized, these techniques revealed that *f*HER2 is overexpressed in 33% of FMC cases, although *f*HER2 and *f*TOP2A gene amplification could not be observed. Our results support the possibility of using FMC as a natural model for comparative oncology. Additional data obtained may also improve the diagnostics, and consequently the treatment, of this type of tumor in veterinary medicine.

Key words: feline mammary carcinomas (FMC), feline human epidermal growth factor receptor-2 gene homolog (*f*HER2), feline topoisomerase 2 alpha gene homolog (*f*TOP2A), gene amplification, protein overexpression, immunohistochemistry (IHC), *in situ* hybridization (ISH)

Introduction

Feline mammary tumors are the third most common malignancy showing a short overall survival period of <12 months (Sorenmo et al., 2013). These tumors, usually classified as carcinomas, display clinical features and an aggressive phenotype similar to those reported in breast cancer in women overexpressing the human epidermal growth factor receptor-2 (HER2). This transmembrane glycoprotein belongs to the epidermal growth factor receptor family and is involved in a variety of molecular pathways associated with tumor growth and progression. In humans, the accurate assessment of HER2 has become critical for chemoimmunotherapy planning (Vollan & Caldas, 2011). For this, clinical oncologists evaluate HER2 protein levels by immunohistochemistry (IHC), and classify tumors as HER2 positive (HER2+) or HER2 negative (HER2–). Equivocal results, classified as 2+ by IHC, lead to the determination of HER2 gene status by the *in situ* hybridization (ISH) technique (Rampaul et al., 2002; Rosa et al., 2009; Susini et al., 2010). Recent data reveal that about 20–30% of human breast cancer patients display HER2 overexpression, and 80% of them show HER2 gene amplification (Wolff et al., 2007), prompting a therapy based on a humanized anti-HER2 monoclonal antibody (Trastuzumab; Roche, Basel, Switzerland), which improves survival rates (Stebbing et al., 2000; Wolff et al., 2007). Furthermore, topoisomerase II alpha gene (TOP2A) is also amplified in 40% of HER2+ breast cancer patients, encouraging a HER2/TOP2A combined inhibitory therapy (Tanner et al., 2006; Fountzilas et al., 2012; Wang et al., 2012).

Recent studies have shown a strong association between feline HER2 (*f*HER2) oncogene homolog overexpression and mammary carcinogenesis, although its biological role remains unclear. Further studies on *f*HER2 protein overexpression incidence are needed, as divergence of the reported data in feline mammary carcinomas (FMC) vary from 5 to 92% (De Maria et al., 2005; Millanta et al., 2005; Wiston et al., 2005; Ordás et al., 2007; Rasotto et al., 2011).

This study aims to optimize *f*HER2 immunodetection in feline tumor samples using different antigen retrieval methods and testing five commercial antibodies. In addition, we assess *f*HER2 and *f*TOP2A gene status in FMC-HER2+ samples using six commercial hybridization DNA probes and verify whether the gene amplification phenomenon is conserved between human and feline species. To achieve comparable data, the American Society of Clinical Oncology (ASCO) guidelines were followed. In particular, sample fixation time and immunohistochemical and fluorescence *in situ* hybridization (FISH) HER2 scoring criteria

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Table 1. Summary of the IHC Protocols Used for *f*HER2 Detection.*

	Primary Antibody		
Clone	Dilution(s)	Incubation Time (min)	Antigen Retrieval Method
A0485	1:250 1:300	60	Boiling water bath (95°C) for 30 min or 60 min with citrate buffer solution (pH 6.0)
CB11	RTU	60	*or*
4B5	RTU	60	Pressure chamber at 2 atm during 2 min with citrate buffer solution
SP3	1:100	60	(pH 6.0)
TAB250	1:50 1:250	60	Boiling water bath (95°C) for 30 min or 60 min with citrate buffer solution (pH 6.0) *or* Pressure chamber at 2atm for 2 min with citrate buffer solution (pH 6.0) *or* Microwave (850 W for 15 min) in citrate buffer solution (pH 6.0) *or* Microwave (900 W for 5 min plus 600W for 15 min) in Tris-EDTA solution (pH 9.0) *or* Proteinase K for 10 min

*IHC, immunohistochemistry; RTU, ready-to-use.

were used. In our opinion, the conflicting results published by several authors about FMC-*f*HER2+ incidence can be explained by the absence of standardized guidelines for *f*HER2 assessment in evaluation of human breast cancer samples.

This study provides new insights on FMC-HER2+ molecular characterization, confirming FMC as a suitable model for comparative oncology studies (Porrello et al., 2004, 2006).

Materials and Methods

Tumor Sample Collection and Histology

Thirty formalin-fixed, paraffin-embedded (FFPE) FMC tissue samples previously fixed for <72 h were obtained from the Veterinary Pathology Diagnostic Service archives (Veterinary Medicine Faculty, Technical University of Lisbon). A human HER2+ breast cancer sample collected at the Medicine Faculty (University of Lisbon) was used as a positive control. For histological classification, 4 μm sections were stained with hematoxylin and eosin (H&E) and tumors were classified according to the World Health Organization (WHO) criteria (Misdorp et al., 1999) being scored from I to III in the malignancy grade (Elston & Ellis, 1998).

Immunohistochemical Studies

Primary Antibodies

The following commercial anti-HER2 antibodies were tested in feline FFPE sections: rabbit polyclonal (A0485; Dako, Glostrup, Denmark); rabbit monoclonal (4B5; Ventana, Tucson, AZ, USA); mouse monoclonal (TAB250; Invitrogen, Carlsbad, CA, USA); rabbit monoclonal (SP3); and a mouse monoclonal (CB11) both from Zytomed (Berlin, Germany).

Immunohistochemical Technique

For each tumor, a selected area was chosen to guarantee the presence of tumoral cells while avoiding tissue necrosis. Immunohistochemical protocols were performed using 4-μm-thick sections, mounted on microscope slides (Starfrost®; Knittel Glaser, Bielefeld, Germany), and dried at 60°C for one hour. Each slide was deparaffinized and rehydrated in a graded alcohol series, followed by an antigen retrieval step in which the tissue sample was incubated with citrate buffer solution ($NaCH_3COO$, pH 6.0) in a pressure chamber (2 atm, 2 min) or in a water bath (95°C, 30 or 60 min) as described in Table 1. In addition, other antigen retrieval conditions were tested to improve TAB250 antibody specificity: microwave cycling with citrate buffer solution, pH 6.0 (850 W, 15 min), microwave cycling with Tris-EDTA buffer solution, pH 9.0 (900 W, 5 min plus 600 W, 15 min) or enzymatic digestion with Proteinase K (Zymed) for 10 min. After blocking endogenous peroxidase activity (peroxide-block solution; Zytomed) for 10 min, each tissue slide was then incubated with a different anti-HER2 primary antibody at room temperature for 1 h. After two PBS washes, the primary antibodies were detected by a 30 min incubation of the HRP-polymer-enhanced IHC detection system (HER2*easy* kit IHC; Zytomed) with 3,3′-diaminobenzidin-tetrahydrochlorid (DAB; Dako), before counterstaining with Mayer's hematoxylin.

A human HER2+ breast cancer slide previously scored as 3+ by IHC (Table 2) was used as the positive control, whereas a human HER2–breast cancer slide scored as 0 (Table 2) was used as the negative control. All the FMC samples were also incubated with either a purified rabbit

Table 2. HER2 IHC Scoring Criteria (HercepTest Interpretation Manual, Dako).

Score	Interpretation
0	No staining
1+	Weak, incomplete membranous staining in any proportion of tumor cells
2+	Complete membrane staining that is either no uniform or weak in intensity but with obvious circumferential distribution in at least 10% of cells
3+	Uniform intense membrane staining of at least 10% of invasive tumor cells

polyclonal IgG (ab27472; Abcam, Cambridge, UK) or a purified mouse polyclonal IgG (ab37355; Abcam) instead of the anti-HER2 primary antibodies, in order to discard species cross-reactivity.

fHER2 Interpretation Criteria

Tumor *f*HER2 positivity was determined on the basis of the maximum area of staining intensity according to the Dako/ASCO guidelines (Table 2), in which *f*HER2 overexpression is defined as a strong membranous staining in more than 10% of the neoplastic cells. FMC samples scored as 0 or 1+ were considered negative, whereas those scored as 2+ or 3+ were considered HER2+. Cytoplasmic staining was considered as nonspecific.

All slides were submitted to blind scoring by two independent pathologists, and discordant interpretation was further debated and settled using a multiviewer microscope.

ISH Studies

DNA Probes

The *f*HER2 and *f*TOP2A gene status evaluation in FMC tissue samples was performed by FISH, chromogenic *in situ* hybridization (CISH), and silver *in situ* hybridization (SISH). FISH was tested using: a Vysis TOP2A/HER2/CEP17 FISH Probe (Vysis; Downers Grove, USA), a ZytoLight SPEC HER2/TOP2A/CEN17 Triple Color Probe (Zytovision, Bremerhaven, Germany), and a HER2 FISH pharmDx kit (Dako). CISH was done with a SPOT-Light HER2 DNA Probe (Zymed) and a Zyto*Dot* SPEC HER2 Probe (Zytovision). Finally, SISH was performed with an Inform HER2 Dual ISH DNA Probe Cocktail (Ventana).

ISH Technique

Optimization of ISH was performed using four FMC-HER2+ cases scored as 3+ by IHC. For this purpose, 23 different protocols were tested to determine *f*HER2/*f*TOP2A gene status variability under different pretreatment conditions, enzymatic digestions, commercial probes, stringent wash, and incubation periods to different immunodetection systems. After optimization, all FMC-HER2+ samples were investigated using the following protocol: tissue sections were dewaxed in two 10-min changes of xylene, rehydrated through two 3-min changes of 100% ethanol, one 3-min change of 95% ethanol, one 3-min step of 70% ethanol, and immersed for 3 min in distilled water. The slides were placed in wash buffer solution (K5599; Dako) in two 3 min changes before the pretreatment, which was performed by microwave cycling (180 W) in four 5-min exposures using 2-[*N*-morpholino]ethanesulphonic acid buffer (MES buffer; Dako). After cooling for 15 min, the slides were placed in wash buffer solution (K5599; Dako) in two 3-min changes, and then incubated in a pepsin proteolytic solution (K5599; Dako) at 37°C for 3–5 min. Then the tissue sections were briefly washed with wash buffer solution for two 3-min changes, immersed in distilled water, dehydrated in ascending alcohol concentrations, and air-dried. A measure of 10–20 μL of the probe was applied per slide according to the size of the tissue and covered with a sealed coverslip. Tissue sections were placed in a Dako Hybridizer (model S2451), which was used to perform DNA denaturation (75°C for 10 min) and hybridization (overnight at 37°C). Thereafter, slides were washed for 5 min in post-hybridization buffer (2×SSC/0.3% NP-40 detergent) at room temperature to remove the coverslips and placed in post-hybridization buffer at 37°C for 5 min. After a brief rinse in wash buffer solution, slides were dehydrated, air-dried (protected from direct light), counterstained with DAPI/Vectashield mounting medium (Vector Laboratories, Peterborough, UK), and covered with a coverslip for observation.

Table 3. HER2 and TOP2A Gene Amplification Assessment (ASCO Guidelines, Tanner et al., 2006; Wolff et al., 2007).

Average Counting of HER2 or TOP2A Copy Number for Cell Nucleus (HER2/CEP17 or TOP2A/CEP17 Ratio)	Interpretation
<4 (<1.8)	Negative for HER2 or TOP2A gene amplification
4.0–6.0 (1.8–2.2)	Equivocal range of values (need of additional cell count or retest)
>6.0 (>2.2)	Positive for HER2 or TOP2A gene amplification

Interpretation Criteria

*f*HER2 and *f*TOP2A gene amplification in FMC tissue samples were evaluated using a confocal laser point-scanning microscope (LSM 710Axio Observer; Carl Zeiss Inc., Jena, Germany). Settings for the individual fluorophores were as follows: 4′,6-diamidino-2-phenylindole (DAPI and centromeric region of human chromosome 17), excitation at 418 nm and recording at 445–480 nm; fluorescein isothiocyanate (FITC for HER2 gene detection), excitation at 503 nm and recording at 520–550 nm; rhodamine (TOP2A gene detection), excitation at 547 nm and recording at 560–600 nm. Tissue areas with nonoptimal enzymatic digestion (poor nuclear resolution or persistent autofluorescence) were rejected and slides were blind-scored by two microscopists. Tumor samples with an average of >6 HER2 gene copies per cell or a ratio between HER2/CEP17 (or TOP2A/

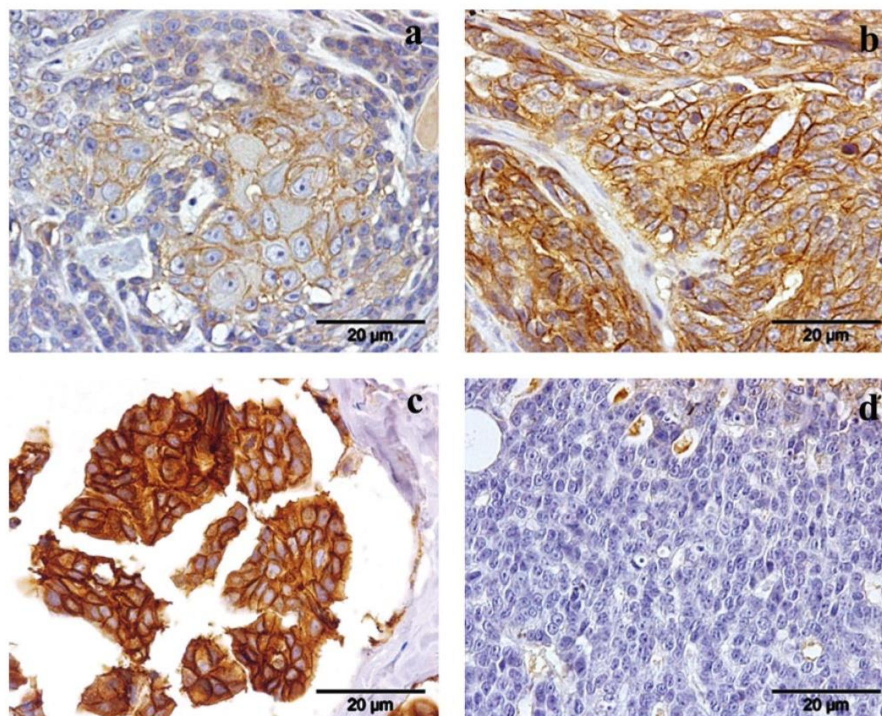

Figure 1. Evaluation of *f*HER2 by IHC: (**a**) *f*HER2 overexpression in a squamous mammary carcinoma scored as 2+; (**b**) FMC-HER2+ cribiform classified as 3; (**b**) human positive control showing an intense and uniform membrane labeling scored as 3+; (**c**) human HER2—breast cancer used as negative control. All samples were counterstained with Mayer's hematoxylin.

CEP17) above 2.2 were considered HER2+, according to the ASCO guidelines for human samples (Table 3). Data sets were acquired by Zen software (Blue Edition, Carl Zeiss) and images were subsequently processed with Adobe Photoshop CS6 software (Adobe Systems Inc., San Jose, CA, USA).

Results

*f*HER2 Overexpression Evaluation in FMC

From the five antibodies tested, CB11, 4B5, and A0485 revealed specific and strong cellular membrane labeling in some FMC samples (Figs. 1a, 1b). Neither TAB250 nor SP3 antibodies showed any species cross-reactivity. From all of the different protocols tested, the best results were obtained whenever extreme antigen retrieval conditions were performed (Table 4). Indeed, when the A0485 polyclonal antibody was associated with the longer AR method (water bath at 95°C for 60 min), the best *f*HER2 protein recognition was achieved (33% of the FMC cases were classified as *f*HER2+). CB11 and 4B5 antibodies gave similar results, particularly when AR was performed in the pressure chamber (8 and 6 FMC-HER2+representing 26.7 and 20%, respectively). As expected, the human positive control showed an intense and uniform membrane labeling (scored as 3+, Fig. 1c), whereas the human negative control did not show any staining (Fig. 1d). The feline negative control slides demonstrated the specificity of the membrane staining as no labeling could be detected.

ISH Study

The ten FMC samples scored as 2+ or 3+ by IHC were subjected to ISH analysis in order to verify whether *f*HER2 overexpression is correlated with *f*HER2 gene amplification. After the protocol optimization, neither *f*HER2 nor *f*TOP2A presented gene amplification, as only one or two signals for each gene could be detected per nucleus (Fig. 2a). Attending to the species sequence specificity, the centromeric region of the feline chromosome E1 could not be detected (Fig. 2a) by using DNA probes recognizing the centromere of the human homologous chromosome 17 (Fig. 2b).

Discussion

In the last decade, efforts have been made to standardize the different molecular techniques to assess HER2 status in humans, in order to achieve reliable and reproducible re-

Table 4. Results of IHC Using A0485, CB11, and 4B5 as Primary Antibodies.8

*f*HER2 Score						
Antibody	AR Method	0	1+	2+	3+	Total
A0485	WB1	16 (53.3%)	5 (16.7%)	8 (26.7%)	1 (3.3%)	
	WB2	15 (50%)	5 (16.7%)	6 (20%)	4 (13.3%)	
	PC	6 (20%)	16 (53.3%)	5 (16.7%)	3 (10%)	
CB11	WB1	24 (80%)	6 (20%)	0 (0%)	0 (0%)	
	WB2	18 (60%)	10 (33.3%)	2 (6.7%)	0 (0%)	30 (100%)
	PC	9 (30%)	13 (43.3%)	6 (20%)	2 (6.7%)	
4B5	WB1	23 (76.7%)	6 (20%)	1 (3.3%)	0 (0%)	
	WB2	15 (50%)	10 (33.3%)	5 (16.7%)	0 (0%)	
	PC	16 (53.3%)	8 (26.7%)	4 (13.3%)	2 (6.7%)	

*IHC, immunohistochemistry; AR, antigen retrieval; WB1, water bath at 95°C with citrate buffer solution for 30 min; WB2, water bath at 95°C with citrate buffer solution for 60 min; PC, pressure chamber at 2 atm with citrate buffer solution for 2 min.

sults (Ménard et al., 2000; Gancberg et al., 2002; Bilous et al., 2003; Wolff et al., 2007; Rosa et al., 2009). Currently, IHC and FISH are the two main techniques routinely performed in human oncology, with well-defined criteria that include pre-analytic conditions (type and time of fixation), analytic indicators (scoring definitions), and post-analytic conditions (interpretation criteria) (Bilous et al., 2003; Wolff et al., 2007).

The results obtained in this study show that *f*HER2 is overexpressed in 33.3% of FMC cases, whenever the A0485 antibody was associated with the longer antigen retrieval protocol (water bath at 95°C for 60 min), similar to results previously published (De Maria et al., 2005; Ordás et al., 2007) and to the incidence reported in women (Wolff et al., 2007).

In the course of this study, the results varied either with the use of different primary antibodies or protocol conditions, and this outcome is congruent with previously published data (one study indicated 5.5%, whereas another referred 92.5% of *f*HER2 positive cases). In fact, CB11 and 4B5 antibodies demonstrated an inferior sensitivity to identify *f*HER2, when compared with A0485 antibody (with 26.7 and 20% of positive cases, respectively), whereas SP3 and TAB250 antibodies did not show any labeling. The absence of labeling with these antibodies, which recognize the HER2 extracellular domain, could be due to the deleterious fixation effects of formaldehyde or differences between HER2 human extracellular domain and *fHER2*, which have a homology of 94%, whereas A0485, CB11, and 4B5 recognize the intracellular domain (O'Malley et al., 2001; Sidoni et al., 2006; Ricardo et al., 2007; Nassar et al., 2009). Altogether, these discrepancies indicate the importance of the technique standardization in order to obtain accurate results.

The analysis of *f*HER2 and *f*TOP2A gene status was only possible by using one of five commercial probe mixtures, probably because *f*HER2 and the human HER2 genes share 88% sequence homology (De Maria et al., 2005; Santos et al., 2012). None of the FMC-HER2+ tumor samples displayed *f*HER2 and/or *f*TOP2A gene amplification, denoting that the oncogenic mechanism responsible for *f*HER2 overexpression is different from the one reported in human HER2+ breast tumor (Ménard et al., 2000; Cianciulli et al., 2002).

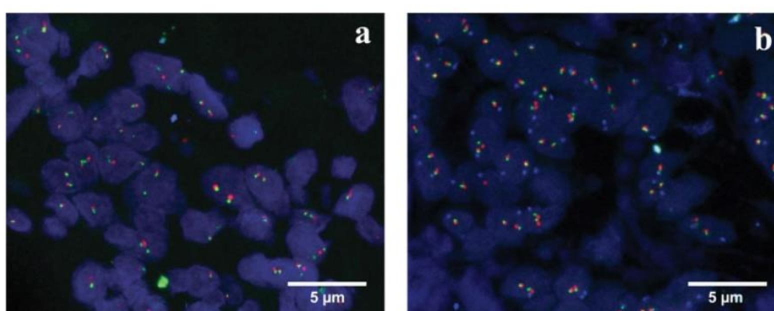

Figure 2. *f*HER2 and *f*TOP2A genes were not amplified in FMC-HER2+: **(a)** *f*HER2 and *f*TOP2A detection by FISH with *f*HER2 gene (green signal) and *f*TOP2A gene (red signal) were not amplified in FMC samples; **(b)** human HER2—breast cancer showing HER2/TOP2A non-amplified genes and the alpha satellite centromeric region of the chromosome 17 (blue signal).

Table 5. IHC and FISH Optimized Protocols for HER2 Detection in Feline Mammary Carcinomas.*

Technique	Protocol Steps			
	Antigen Retrieval Method	Antibody		
IHC		Clone	Dilution	Incubation Time
	WB	A0485	1:300	60 min
	Pretreatment	Enzymatic Digestion	DNA Probe	Post-Hybridization
FISH	MW with MES buffer (4 × 5 min)	Pepsin proteolytic solution (37°C) for 3–5 min	ZytoLight SPEC HER2/ TOP2A/CEN17 Triple Color Probe	SSC/NP-40 buffer, 5 min

*IHC, immunohistochemistry; WB, water bath at 95°C with citrate buffer solution for 60 min; FISH, fluorescent *in situ* hybridization; MW, microwave cycling (180W); MES, 2-[*N*-morpholino]ethanesulphonic acid buffer; SSC/NP-40, 2 × SSC/0.3% NP-40 detergent.

Nevertheless, our data are in agreement with the results reported by the unique study that evaluated *f*HER2 gene status in FMC (Ordás et al., 2007).

In addition, *f*TOP2A gene amplification was not detected in FMC-HER2+ samples, in accordance with what has been described in human HER2+ breast cancer cases. The TOP2A gene is known to be rarely amplified when the HER2 gene is not (Bhargava et al., 2005; Oakman et al., 2009). The absence of *f*TOP2A gene amplification raises questions about the beneficial effects of using doxorubicin-based chemotherapy in FMC (McNeill et al., 2009; Sorenmo et al., 2013).

In feline oncology, mammary tumors are usually highly aggressive, growing rapidly and metastasize to regional lymph nodes and the lungs, sharing several clinical features with the human breast cancer HER2+ subtype. This enhances the possibility of using cats as a breast cancer model (De Maria et al., 2005).

Conclusions

In summary, our results indicate that *f*HER2 protein and gene status evaluation should be performed following the optimized protocols described in Table 5. The improved IHC protocol revealed that *f*HER2 is overexpressed in about 33% of FMC cases, highlighting the relevance of immunostaining standardization in veterinary diagnosis. The evaluation of *f*HER2 and *f*TOP2A gene status demonstrated that neither of these genes is amplified in FMC-HER2+using the FISH technique, raising doubts about the efficiency of anthracycline-based chemotherapy regimens in cat.

In addition, our results strongly suggest the possibility of using FMC-HER2+ as a suitable cancer model to study human HER2+ breast carcinomas without gene amplification.

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ANNEX II – Ki-67 as a Prognostic Factor in Feline Mammary Carcinoma: What Is the Optimal Cutoff Value?

Oncology–Original Article

Ki-67 as a Prognostic Factor in Feline Mammary Carcinoma: What Is the Optimal Cutoff Value?

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Abstract

Ki-67 is a nuclear protein and a proliferation marker frequently used in establishing the prognosis for breast cancer patients. To investigate the prognostic value of the Ki-67 proliferation index in female cats with mammary carcinoma, a prospective study was conducted with 96 animals. The Ki-67 index of primary tumors ($n = 96$) was initially determined, and whenever possible, the Ki-67 index of regional lymph node metastasis ($n = 38$) and distant metastasis ($n = 16$) was also estimated. The optimal cutoff value for the Ki-67 index was determined by univariate and multivariate analysis. Ki-67 indices ≥14% were detected in 72.9% (70 of 96) of the tumors. Tumors with a Ki-67 index ≥14% were significantly associated with large size ($P = .022$), poor differentiation ($P = .009$), presence of necrotic areas ($P = .008$), estrogen receptor-negative status ($P < .0001$), *f*HER2-negative status ($P = .003$), and shorter overall survival ($P = .012$). Moreover, Ki-67 expression in the primary tumor was strongly and positively correlated with both regional metastasis ($P < .0001$; $r = 0.83$) and distant metastasis ($P < .0001$; $r = 0.83$), and was significantly higher in distant metastases when compared with the primary tumor ($P = .0009$). A similar correlation was also observed between regional and distant metastasis ($P < .0001$; $r = 0.75$). On the basis of the above results, the authors propose the adoption of the 14% value as the optimal cutoff for Ki-67 to identify tumors with high risk of disease progression.

Keywords

feline mammary carcinoma, Ki-67 proliferation index, prognostic factor, cutoff value

Feline mammary carcinomas (FMCs) are among the most prevalent tumors in cats, with an incidence that can reach 40% of all tumor cases in this species.[23,39,42] Contrary to those of humans and dogs, 85% to 93% of feline mammary tumors are considered malignant, and the most common histotypes are the tubular, papillary, solid, and cribriform carcinomas.[39] Feline mammary tumors may metastasize to the regional lymph nodes, lungs, and liver, as well as other organs. Although some studies correlate age, tumor size, presence of regional metastasis, and malignancy grade with overall survival (OS),[19,37,43] still scarce information is available regarding the proliferation index of FMC. Based on this scenario, additional prognostic and predictive factors may provide useful insights into tumor biology to achieve a more efficient therapy and a better follow-up of the affected female cats.

Ki-67 is a protein found only in growing, dividing cells. It is expressed in all cell cycle phases, except in the resting phase (G0). With an intranuclear localization, the expression levels of Ki-67 are low during the G1 and S phases, rapidly increase during the G2 phase, and reach a peak in mitosis. This well-defined expression pattern makes the Ki-67 antigen a good proliferation marker, very useful and reliable in the prognosis of human breast cancer.[9,45] Several studies of women demonstrated that high Ki-67 expression is a poor prognostic indicator of 5-year recurrence-free survival in breast cancer patients,[6,7,9,18,27,41] being also used for chemotherapy response prediction.[6,9,18] However, different Ki-67 cutoff values and tumor heterogeneity require an adaptation of the scoring system, with cutoff values varying from 3.5% to 34%.[7,9,14,29] To overcome this problem, a large study recently recommended the standardization of a Ki-67 cutoff point at 14% in breast cancer to improve prognosis.[5]

In veterinary medicine, although the Ki-67 index has been investigated as a prognostic factor in FMC, few data are

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available on how the Ki-67 index correlates with lower survival.[23,32,37] Furthermore, the evaluation of cell proliferation by Ki-67 immunostaining has not been subject to standardization of protocols. The lack of clear indication on a cutoff level explains why no values have been validated for FMC.[4,30,32,37]

The main goal of the present prospective study is to validate the prognostic value of the Ki-67 index in FMC. First, the Ki-67 index of 96 primary mammary tumors was determined and compared with corresponding lymph node and distant metastasis. Second, the ideal cutoff value was calculated by evaluating a set of threshold values based on univariate and multivariate survival analysis. Finally, the association between the Ki-67 index and 19 other clinicopathologic features of cats with mammary carcinoma was evaluated.

Table 1. Univariate and Multivariate Analysis of Ki-67 Proliferation Index.

Univariate: Ki-67	Animals, n	OS Time (months), Mean ± SD	95% CI	*P*
Cutoff of 14%				.012
Low, <14%	23	33.32 ± 3.61	26.2, 40.4	
High, ≥14%	66	22.55 ± 2.80	17.1, 28.0	
Cutoff of 16%				.019
Low, <16%	31	30.31 ± 3.26	23.9, 36.7	
High, ≥16%	58	22.28 ± 3.01	16.4, 28.2	
Cutoff of 17%				.027
Low, <17%	33	31.77 ± 3.58	24.8, 38.8	
High, ≥17%	56	22.17 ± 3.18	15.9, 28.4	
Multivariate: Ki-67		**HR**	**95% CI**	***P***
Cutoff of 14%		2.4	1.1, 5.1	.027
Cutoff of 16%		—	—	.436
Cutoff of 17%		—	—	.115

Abbreviations: CI, confidence interval; HR, hazard ratio; OS, overall survival.

Materials and Methods

Animal Population

The studied population consisted of 96 female cats with mammary tumors admitted to the Small Animal Hospital of the Faculty of Veterinary Medicine of the University of Lisbon from June 2011 to December 2013. Animals were followed for at least a month to be included in survival analysis, and the last follow-up was in May 2014. For each animal, the following clinical data were recorded: age, breed, reproductive status (intact vs spayed), administration of progestogens, number and location of tumors, tumor stage (TNM system),[39] treatment prescribed (none, mastectomy, mastectomy combined with chemotherapy), and OS. Additionally, the presence of microscopic regional lymph node metastasis was evaluated in a majority of cases (86 cats). A full postmortem examination was performed in 18 cats, and metastatic disease was confirmed histologically in 16 of those animals.

All mammary and respective metastatic lesions excised during surgery from 89 cats or during necropsy (7 cats) were fixed in 10% buffered formalin for 24 to 48 hours and embedded in paraffin. The histologic specimens were evaluated following the World Health Organization classification system.[24] The degree of malignancy was determined by the assessment of 3 morphologic features (tubule formation, nuclear pleomorphism, and number of mitosis)[10] and scored from I (well-differentiated tumor) to III (poorly differentiated tumor), according to the Elston and Ellis grading system. Detailed information was also collected about tumor size, histopathologic classification, malignancy grade, presence of necrotic areas, lymphatic invasion by neoplastic cells, lymphocytic infiltration, and skin ulceration.

Immunohistochemistry

The Ki-67 proliferation index, the status of estrogen receptor (ER) and progesterone receptor (PR), and the expression of feline epidermal growth factor receptor type 2 (*f*HER2) and cytokeratin 5/6 (CK 5/6) were evaluated in all mammary carcinomas ($n = 213$), lymph node metastasis ($n = 59$), and distant metastasis ($n = 69$) collected during surgery or necropsy. A representative area of each lesion (diameter, 0.6 cm) was selected and used to prepare 5 serial 3-μm sections, which were later attached to SuperFrost Plus microscope slides (Thermo Scientific, Rockford, IL, USA). Tissue sections were then dried at 60°C for 1 hour and deparaffinized. The antigen retrieval was performed by boiling samples for 2 minute in a sodium citrate buffer solution (0.01M $NaCH_3COO$, pH 6.0) using a pressure cooker (2 atm).[3,38] For PR immunodetection, antigen retrieval was achieved with the same sodium citrate buffer in a water bath for 60 minutes at 95°C. For CK 5/6, samples were microwaved at 900 W for 15 minutes in Tris-EDTA buffer (pH 9.0).[2]

Immunohistochemical analysis was performed with the primary antibodies summarized in Supplemental Table 1, and staining was achieved with a modified streptavidin–peroxidase conjugate method (Novolink MaxPolymer Detection System, Leica Biosystems, Wetzlar, Germany). Finally, tissue sections were counterstained with Mayer's hematoxylin (Merck, NJ, USA).

Feline tonsil was used as positive control for Ki-67 index, while normal skin was used as a positive control for CK5/6 expression. Feline mammary samples with known positive and negative ER, PR, and *f*HER2 status were also employed as positive controls.

Immunohistochemistry staining was scored by 2 independent observers, and discordant interpretations were settled using a multiviewer microscope. Tumor cells were considered Ki-67 positive in the presence of brown nuclear staining of granular or diffuse type.[5,9,30,45] The Ki-67 proliferation index was determined by assessing the percentage of positively staining tumor cell nuclei in 1000 tumor cells. For each lesion, 5 to 6 images were randomly taken with an Olympus DP25 camera (Pennsylvania, USA) on an Olympus BX51 light microscope and analyzed using Image J (Open Source Software, version 1.46r; National Institutes of Health, Bethesda, MD, USA).

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ER and PR expression was evaluated in tumor tissues using the Allred score system[15,25] (Suppl. Table 2), where a score ≥3 is considered positive.[13] *f*HER2 staining intensity was evaluated by using the Food and Drug Administration–approved scoring system,[13,44] in which tumors with immunohistochemistry scores of 3+ (uniform and intense membrane staining of at least 10% of tumor cells) or 2+ (complete membrane staining, not uniform or weak in intensity but with obvious circumferential membrane distribution in at least 10% of cells) were considered HER2 positive.[2,3,21,35,38] Finally, tumors were considered CK 5/6 positive when >1% of cells were immunoreactive.[1,2,16]

Determination of the Optimal Cutoff Value for Ki-67 Index

Whenever a cat exhibited multiple mammary tumors, the lesion with higher risk of malignancy was selected for further statistical analysis based on tumor size and malignancy grade, which both have been associated with worse prognosis in FMC.[19,37,43]

After testing for normality, the paired *t* test was applied to make a comparison between the Ki-67 index of primary tumors and regional metastasis, between the Ki-67 index of primary tumors and distant metastasis, and between the Ki-67 index of regional and distant metastasis. Correlation analyses were performed by using the Pearson correlation test. When multiple metastases were present (regional and/or distant), the lesions with higher Ki-67 expression were chosen for statistical analysis.

To determine the optimal cutoff value for Ki-67 in FMC, an univariate survival analysis was performed testing the following range of values, also used in human breast cancer patients: 5%, 10%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 25%, 30%, and 35%.[7,9,29] Regarding the survival analysis, OS was defined as the time, in months, between the initial diagnosis and death or the date of the last follow-up for surviving cats (censored observations). Only deaths attributed to mammary carcinoma progression were considered. In this study, 2 deaths were caused by other diseases (censored observations), both belonging to the group of cats with high Ki-67 indexes: one was caused by renal failure, and the other animal was euthanized because owners could not afford the proposed surgery. Among the total of the cats in the study ($n = 96$), 7 were excluded for survival analysis, once they were followed for less than a month. OS curves were estimated using the Kaplan-Meier method and compared with the log-rank test.

The cutoff values that showed significance were subjected to multivariate analysis using the Cox proportional hazard model. The hazard ratio was calculated with a 95% confidence interval (95% CI).

After the establishment of the Ki-67 cutoff, the positive predictive value (PPV) and negative predictive value (NPV) were calculated for predicting death and survival after 1 and 2 years after the surgery. For this analysis, only the animals subjected to surgery were considered, with those that also received chemotherapy being excluded.

The Fisher exact test was used to assess the association between Ki-67 expression and 19 clinicopathologic features: age, breed, reproductive status, previous administration of progestogens, tumor number, location and size, stage of disease, histopathologic classification, malignancy grade, presence of necrotic areas, lymphatic invasion, lymphocytic infiltration, cutaneous ulceration, regional lymph node metastasis, receptor status (ER, PR, *f*HER2), and CK 5/6 expression. For each significant association, odds ratio (OR) was calculated with a 95% CI.

The association between Ki-67 labeling index and tumor size was analyzed after dividing the population into 3 subgroups, according to the tumor size, using the levels established in the TNM classification system (<2 , 2–3 , and >3 cm). To evaluate the effect of age, the population was split into 3 subgroups: <8, 8–12, and >12 years old.

Quantitative data were processed and analyzed with SPSS 21.0 (IBM, New York, NY, USA), and a 2-tailed $P < .05$ was considered statistically significant.

Results

Animal Population Data

Ninety-six female cats with mammary carcinoma were followed up, and their clinicopathologic features are summarized in Supplemental Table 3. The mean ± SD age of the animals was 11.49 ± 2.85 years (range, 5–19 years). The majority of the cats were subjected to surgery ($n = 89$), including unilateral mastectomy in 71 (79.8%), regional mastectomy in 11 (12.4%), and bilateral mastectomy in 7 (7.8%). Eight mastectomized cats received anthracycline-based chemotherapy after surgery (doxorubicin, 25 mg/m^2, intravenously, every 3 weeks for 5 cycles).[39]

In this study, 213 mammary carcinomas were collected from the 96 queens. In the 60 animals that showed >1 tumor, the most malignant lesion was chosen (see Materials and Methods). The mean size of the tumors was 2.71 ± 1.5 cm (range, 0.5–7 cm).

By the end of the study, 48 animals had died or were euthanized due to metastatic progression of the disease, and for those animals, the median OS was 10.5 months (mean, 12.2 months).

Ki-67 Index of the Primary Tumor Is Strongly and Positively Correlated With the Ki-67 Index of Regional and Distant Metastasis

The immunohistochemical analysis of the Ki-67 expression revealed a moderate to strong nuclear staining of tumor cells (Fig. 1a), especially in distant metastases (Fig. 1b). The mean Ki-67 index was 26% ± 1.8% in primary tumors (range, 0.2%–85.8%; median, 21.4%), rising to 30.1% ± 3.1% in regional lymph node metastases (range, 3.5%–78.8%; median, 26.4%, $n = 38$). Distant metastases ($n = 16$) showed a mean Ki-67 index of 48.2% ± 5.1% (range, 13.3%–92.1%; median, 44.6%).

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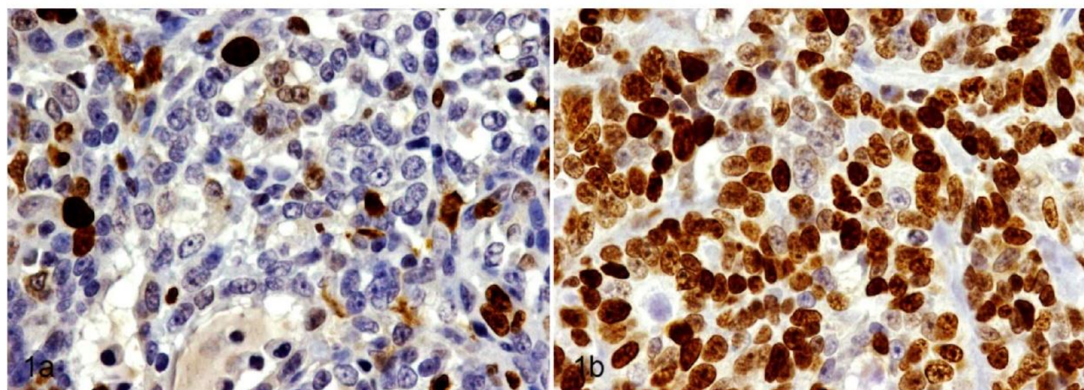

Figure 1. Cribriform carcinoma, mammary gland, cat. Nuclear Ki-67 staining. (a) Primary tumor with a Ki-67 index of 13.7%; (b) lung metastasis with a Ki-67 index of 88.6%. Immunohistochemistry for Ki-67.

A strong and positive correlation was found between the Ki-67 index of the primary tumor and regional metastasis ($P < .0001$; $r = 0.83$, $n = 38$), between the Ki67 index of the primary tumor and distant metastasis ($P < .0001$; $r = 0.83$, $n = 16$), and between the Ki-67 index of regional and distant metastasis ($P = .0052$; $r = 0.75$, $n = 16$).

In addition, the Ki-67 index of primary tumors was significantly lower when compared to the Ki-67 index of distant metastasis ($P = .0009$, $n = 16$). Differences between regional and distant metastasis approached significance ($P = .08$, $n = 16$), with distant lesions showing higher Ki-67 scores. Finally, primary tumors and regional metastasis presented distinct Ki-67 scores, which were not statistically significant ($P = .186$, $n = 38$).

The Optimal Cutoff Value of Ki-67 Index Is 14% in FMC

To determine the optimal cutoff point for the Ki-67 index, a univariate survival analysis was performed using different cutoff values (5%, 10%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 25%, 30%, and 35%). Statistically significant differences in OS curves were observed only for the cutoff of 14% ($P = .012$), 16% ($P = .019$), and 17% ($P = .027$; Table 1, Fig. 2). Further multivariate analysis showed that a 14% cutoff value could better distinguish cats with highly malignant mammary carcinomas from those with low malignant potential. In addition, only a cutoff value of 14% was identified to be a Ki-67 independent prognostic indicator of OS ($P = .027$), showing a hazard ratio of 2.4 (Table 1).

The majority of the animals that presented low Ki-67 values were alive by the end of the follow-up period (69.6%, 16 of 23), while only 37.9% (25 of 66) of cats with higher Ki-67 values (≥15%) were alive. Moreover, most of the cats that died had a primary tumor with a Ki-67 index ≥14% (85.4%, 41 of 48), and only 7 presented low Ki-67 values (14.6%, 7 of 48).

Animals With High Ki-67 Index (>14%) Have Higher Probability of Dying Within the 2 Years After Surgery

With the 14% as a threshold value, the PPV and NPV were calculated for predicting death or survival at 1 and 2 years after surgery. For 1 year after surgery, the PPV for predicting death was 50%, and the NPV was 81.8%. For 2 years of survival after surgery, the PPV was 80%, and the NPV was 27.3%.

Ki-67 Index ≥14% Is Significantly Associated With Unfavorable Features

Of the 96 cats with mammary carcinoma, 70 showed mammary tumors with a Ki-67 index ≥14% (72.9%), while 26 cats showed lower Ki-67 indices (27.1%). The majority of the cats with mammary carcinoma presenting with regional lymph node metastasis had a Ki-67 index ≥14% (78.9%, 30 of 38). Of 16 metastatic disease cases, 15 (93.8%) also had distant metastasis with a Ki-67 index ≥14%.

Moreover, primary mammary carcinomas that showed a Ki-67 index ≥14% were significantly associated with large size ($P = .022$; OR, 4.4; 95% CI: 1.12, 21.65), poor differentiation (grade III, $P = .009$), presence of necrotic areas ($P = .008$; OR, 3.7; 95% CI: 1.25, 11.13), negative ER status ($P < .0001$; OR, 7.12; 95% CI: 2.38, 22.66), and low *f*HER2 expression ($P = .003$; OR, 4.31; 95% CI: 1.47, 12.98; Supplemental Table 4). In contrast, no significant statistical association was found between Ki-67 index ≥14% and age, breed, reproductive status, previous administration of progestogens, tumor location, multiple mammary tumors, lymph node metastasis, disease stage, histopathologic classification, lymphatic invasion by neoplastic cells, lymphocytic infiltration, skin ulceration, and PR or CK5/6 status of the primary tumor (Suppl. Table 4).

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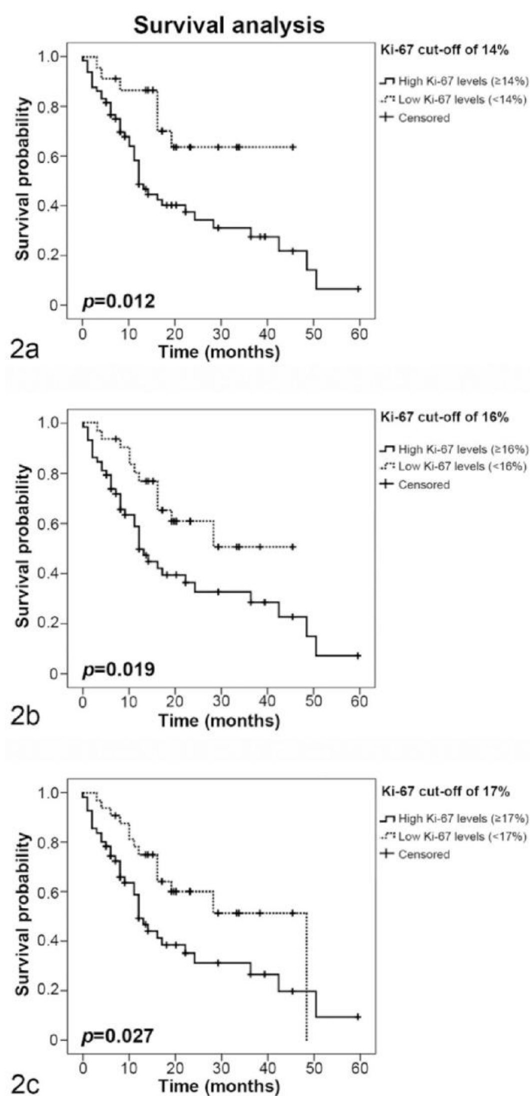

Figure 2. Kaplan-Meier overall survival curves using different cutoff values of Ki-67. Overall survival curves with Ki-67 cutoff values of 14% (a), 16% (b), and 17% (c). In all scenarios, cats with feline mammary carcinoma showing a Ki-67 index below the cutoff value survived significantly longer than those with higher Ki-67 indexes.

Discussion

Until now, the World Health Organization histopathologic classification has been extensively used to establish the prognosis in cats and dogs with mammary tumors.[10,24,39] Although tumor cell proliferation has emerged as an important diagnostic and prognostic tool in human breast cancer patients,[5,9–11,17,28,31,36] no standard cutoff point for the Ki-67 index is known in FMCs.[37] Recently, some studies assessed Ki-67 expression in feline mammary tumors and showed that malignant lesions exhibited higher Ki-67 levels that benign lesions.[3,4,23,32,33,37] In the present study, we demonstrated that the Ki-67 labeling index in the primary tumor reveals a strong and positive correlation with the Ki-67 index of local and distant metastasis. Moreover, and besides the small sample ($n = 16$), the metastatic lesions had significantly higher Ki-67 indices than the primary lesions (means of 48.2% vs 26%), corroborating the association between Ki-67 overexpression in the primary tumor and aggressive tumor behavior.

Employing the methodology used in human clinical studies,[27,29] the optimal cutoff value for Ki-67 was determined. The Ki-67 cutoff of 14% was considered as the optimal value to predict clinical outcome of FMC. Indeed, cats whose primary tumors showed a Ki-67 index ≥14% displayed a 2.4-fold increased risk of tumor-related death, in comparison with cats having mammary tumors that exhibited a lower Ki-67 index (Table 1). Most primary tumors (72.9%) and metastatic lesions (93.8%) showed a Ki-67 index ≥14%, with the corresponding cats presenting significantly shorter survival. In fact, the majority of cats that died from mammary cancer disease during the follow-up showed a primary tumor with a Ki-67 index ≥14% (41 of 48, 85.4%).

The Ki-67 index also demonstrated a strong predictive value, with a high proportion of cats with low levels of Ki-67 alive after 1 year of the surgery (NPV = 81.8%). Moreover, high Ki-67 index levels also demonstrated an important predictive value, as the probability of the cat dying within the 2 years after surgery was 80% (ie, PPV = 80%).

In parallel, we also observed a strong association between Ki-67 levels and several clinicopathologic features associated with shorter OS, such as size of the tumor,[19,43] malignancy grade,[37] presence of necrotic areas,[30,37] and low ER expression,[22] which reinforces the relevance of this marker for the clinical follow-up of FMC.

In human breast cancer, a positive ER status is associated with low cancer mortality.[8,13] Our results corroborate this scenario in FMC. Regarding HER2 tumor status, its overexpression is associated with worse prognosis in breast cancer[20,44]; however, its role in FMC is still controversial. It was reported that overexpression of *f*HER2 is associated with short OS periods.[21] However, a recent report claimed that *f*HER2 overexpression is associated with feline tumors that show nonmalignant features.[35] Our findings support these results, since mammary tumors with Ki-67 levels ≥14% were significantly associated with a negative *f*HER2 status, indicating that *f*HER2 overexpression could be associated with a better clinical outcome.

Recent studies also showed that Ki-67 immunohistochemistry measurements can significantly predict breast cancer outcome in women treated with anthracycline-based chemotherapy.[26,34,40] Anthracyclines are a class of antitumor drugs (eg, doxorubicin) that inhibit a family of enzymes essential for cell division (topoisomerases type II), making these drugs a primary choice in the treatment of tumors with high growth rates, being also used to

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treat a variety of malignancies in cats, including mammary carcinoma.[39] To the best of our knowledge, there are no studies showing that chemotherapy improves survival in cats with mammary carcinomas showing a high Ki-67 index. Unfortunately, in our study, only 8 cats (8.3%) with mammary carcinoma were treated with doxorubicin after surgery, not allowing us to assess the value of Ki-67 in predicting the benefit of anthracycline-based chemotherapy.

In conclusion, our study demonstrated that the Ki-67 index can be used as a prognostic biomarker in cats with mammary carcinoma—specifically, it showed that Ki-67 values $\geq$14% are associated with lower OS and with other aggressive clinicopathologic features. All results presented above suggest that a Ki-67 cutoff of 14% can be regarded as a useful tool to identify animal patients with worse prognosis.

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Declaration of Conflicting Interests

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ANNEX III – Molecular based subtyping of feline mammary carcinomas and clinicopathological characterization

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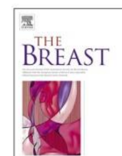

Original article

Molecular based subtyping of feline mammary carcinomas and clinicopathological characterization

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ABSTRACT

Molecular classification of feline mammary carcinomas (FMC) from which specific behavioral patterns may be estimated has potential applications in veterinary clinical practice and in comparative oncology. In this perspective, the main goal of this study was to characterize both the clinical and the pathological features of the different molecular phenotypes found in a population of FMC ($n = 102$), using the broadly accepted IHC-based classification established by St. Gallen International Expert Consensus panel.

The luminal B/HER2-negative subtype was the most common (29.4%, 30/102) followed by luminal B/HER2-positive subtype (19.6%, 20/102), triple negative basal-like (16.7%, 17/102), luminal A (14.7%, 15/102), triple negative normal-like (12.7%, 13/102) and finally, HER2-positive subtype (6.9%, 7/102). Luminal A subtype was significantly associated with smaller tumors ($p = 0.024$) and with well differentiated ones ($p < 0.001$), contrasting with the triple negative basal-like subtype, that was associated with larger and poorly differentiated tumors ($p < 0.001$), and with the presence of necrotic areas in the tumoral lesion ($p = 0.003$). In the survival analysis, cats with Luminal A subtype presented the highest survival time (mean OS = 943.6 days) and animals with triple negative basal-like subtype exhibited the lowest survival time (OS mean = 368.9 days). Moreover, two thirds (64%, 32/50) of the queens with multiple primary tumors showed different molecular subtypes in each carcinoma, revealing that all independent lesions should be analyzed in order to improve the clinical management of animals.

Finally, the similarities between the subtypes of feline mammary tumors and human breast cancer, reveal that feline can be a valuable model for comparative studies.
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Introduction

Feline mammary carcinomas have a deserved reputation of highly malignant behavior with most tumor types being considered similarly as bad news as far as prognosis is considered [1]. However, the considerable variety of histological types and clinical outcomes up rise the concern that there may be some variations that need to be exploited as therapeutic approach may vary from surgical excision and nothing else to combinations of this with chemotherapy. The gain in discriminating between various types of prognosis may be not just beneficial for the female cats and their owners but also for the characterization of an animal population, readily available, that has not been conveniently exploited in translational studies in cancerology. It has long been proven that feline mammary tumors have much more similarities to human mammary tumors, than rodent tumor models [1–3], with some studies suggesting a viral etiology to mammary tumors in both species [4–8] and some emphasizing the zoonotic potential of feline mammary tumor virus [4,9] (Retroviridae).

It is generally accepted that early detection and more effective treatments are key factors that explain longer survival times in human cases of mammary carcinoma [10]. More effective treatments have benefited from advances in tumor classification evolving from systems based upon molecular and immunophenotypic markers. The first molecular classification for breast cancer was proposed by Perou and colleagues (2000), which divided

Abbreviations: FMC, feline mammary carcinomas; IHC, immunohistochemistry; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor-2; CK, cytokeratins; OR, odds ratio; OS, overall survival; DFS, disease free survival; Luminal B/HER2−, Luminal B/HER2-negative; Luminal B/HER2+, Luminal B/HER2-positive; TN, triple negative.

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breast tumors in four groups (luminal-like, basal-like, HER2+ and normal-like), according to their gene expression analysis [11]. The prohibitive cost of this multigenic assay, led to the development of alternatives, such as the evaluation of biomarkers using immunohistochemistry (IHC) analysis, which was suggested as a surrogate for gene expression profiling [12,13]. Recently, the St. Gallen International Expert Consensus panel proposed an IHC-based classification that establishes six biologically distinct breast cancer subtypes: luminal A, luminal B/HER2-negative, luminal B/HER2-positive, HER2-positive (non-luminal), triple negative basal-like and triple negative normal-like [14].

The luminal A is the most common subtype and the one with better prognosis. It is characterized by the overexpression of estrogen and/or progesterone receptor (ER and/or PR), HER2-negative status and low Ki-67 index [14,15]. Luminal B breast tumors display a more aggressive behavior than luminal A and are divided in two subtypes: the luminal B/HER2-negative which show positive staining for ER and/or PR, negative expression for HER2 and high Ki-67 index levels, and the luminal B/HER2-positive subtype that shows ER and/or PR and HER2 overexpression [14,16]. Patients with luminal tumors usually benefit from endocrine therapies. Within the non-luminal subtypes, the HER2-positive subtype is characterized by the HER2 overexpression in the absence of hormone receptors (ER and PR). This subtype is associated to a poor prognosis, but fortunately, specific anti-HER2 therapies have improved the survival rate of patients [15,17,18]. Finally, the triple negative tumors show the worst prognosis of all breast cancer subtypes and are characterized by the lack of ER, PR and HER2 expression. They are classified in basal-like and normal-like tumors, based upon the cytokeratin expression (CK 5/6, 14 and 17), that is positive in the basal-like tumors [19–22].

In Veterinary Medicine, some investigation has been conducted to find biomarkers that could improve the prognosis accuracy in cats with mammary carcinomas [23–33]. This effort is important since feline malignant mammary tumors are very common in cats, representing the third most common tumor in this species. They are predominantly malignant (85%–95%) and clinically very aggressive [1,33,34]. Recently, three studies have used a panel of markers to immunophenotype feline mammary carcinomas (FMC) [35–37]. However, the small number of tumor samples and the use of different classifications led to contradictory conclusions. In this study, we aimed to overcome this difficulty clinically characterizing the different subtypes identified in a large population of female cats presenting mammary carcinomas ($n = 102$), using the IHC-based classification established by St. Gallen International Expert Consensus panel [14]. Significant statistical associations between cancer subtypes and 19 clinicopathological features were evaluated, and a univariate analysis of overall survival (OS) and disease free survival (DFS) was performed.

Table 1
Allred Score guidelines for ER and PR staining.

Score for percentage of positive tumor cells		Score for average intensity of staining	
Score	Interpretation	Score	Interpretation
0	No staining	0	None
1	<1%	1	Weak
2	1–10%	2	Average
3	10–33%	3	Strong
4	33–66%		
5	>66%		

Allred score (0–8) = The Σ of both scores.

Material and methods

Study population

A total of 102 female cats with mammary carcinoma were enrolled in this prospective study from September 2009 to January 2014. Animals were presented at the Teaching Hospital of the Faculty of Veterinary Medicine, University of Lisbon (FVM-ULisboa), and the owners gave permission to collect samples from their pets and to use the animal's clinical data. All mammary tumors were surgically obtained after mastectomy, except for 9 cases that were collected at necropsy. Tumor samples were collected in accordance with the EU Directive 2010/63/EU and research was approved by the Ethics Committee of the FVM-ULisboa.

The following clinical and pathological features were evaluated and recorded: age, breed, reproductive status at the time of the surgery (intact *versus* spayed), previous administration of progestogens for oestrus control, number, location and size of tumor lesions, treatment performed (none, surgery), extension of the surgery (lumpectomy, unilateral mastectomy or bilateral mastectomy), stage of the disease (TNM system) [1], disease free survival (DFS) and overall survival (OS).

Tumor histological classification

Mammary tumors were fixed in 10% buffered formalin during 24–48 hours and were processed for routine histological examination. All tumors were classified according to the World Health Organization (WHO) classification system [38]. The malignancy grade was scored from I to III using the Elston & Ellis scoring system [39] and the presence of necrotic areas within the lesions, lymphatic invasion by neoplastic cells, lymphocytic infiltration and cutaneous ulceration was recorded. In 93 cases, the regional lymph nodes were also collected and analyzed.

Immunohistochemistry

For each primary tumor, a specific area was selected (6 mm in diameter), avoiding the necrotic and the non tumoral areas, sections were obtained and immunohistochemical staining was performed for detection of the following proteins: ER, PR, feline homologue of HER2 (*f*HER2), CK 5/6 and Ki-67. IHC protocols and score interpretation were performed as previously described [32,36,40,41].

Classification of the staining results was made according to the Allred Score guidelines for interpretation of ER and PR staining (Table 1) and HER2 was interpreted according to the American Society of Clinical Oncology (ASCO) guidelines which criteria are summarized in Table 2 [12,42–44].

A tumor was considered positive for ER and PR when presenting a score >2 [32,41,43]; for *f*HER2 when achieving the score 2+ or 3+ [2,25,40]; and for CK 5/6 status, when revealing cytoplasmic and/or membrane labeling of 1% of the tumor cells [35,41,45]. For Ki-67, a

Table 2
*f*HER2 immunohistochemistry scoring criteria.

Score	Interpretation
0	No staining
1+	Weak, incomplete membrane staining in any proportion of tumor cells
2+	Complete membrane staining that is either no uniform or weak in intensity but with obvious circumferential distribution in at least 10% of cells
3+	Uniform intense membrane staining of at least 10% of invasive tumor cells

Table 3
Immunohistochemical definitions of the molecular subtypes in FMC.

	Luminal A	Luminal B		HER2-positive	Triple negative	
		Luminal B/HER2-	Luminal B/HER2+		Basal-like	Normal-like
ER	ER+ and/or	ER+ and/or	ER+ and/or	–	–	–
PR	PR+	PR+	PR+	–	–	–
*f*HER2	–	–	+	+	–	–
Ki-67 index	Low	High	Any	Any	Any	Any
CK 5/6	Any	Any	Any	Any	+	–

tumor was considered highly proliferative when presenting a Ki-67 index ≥14% [32].

For molecular classification of the FMC the St. Gallen International Expert Consensus panel was applied, using five biomarkers (ER, PR, HER2, Ki-67 and CK 5/6), as resumed in Table 3 [13,14,41,46,47].

Statistical analysis

For statistical analysis the Statistical Package for the Social Sciences 20.0 for Windows (SPSS Incorporation, IBM, New York, USA) was used. A two-tailed p value less than 0.05 was considered statistically significant.

Analysis of variance (ANOVA) of the molecular subtypes of FMC was performed for age (in years), tumor size (in centimeters) and Ki-67 proliferation index (in percentage). When significant, differences between pairs of molecular subtypes were evaluated by the Tukey HSD method.

The Fisher's exact test was used to assess any association between the different molecular subtypes and the following clinicopathological features: age breed, reproductive status, administration of progestogens, number, location and tumor size, lymph node status, stage

Table 4
FMC clinical and pathological characteristics and associations with the molecular subtype.

Clinical features	Total (n = 102)	Luminal A (n = 15)	Luminal B/HER2– (n = 30)	Luminal B/HER2+ (n = 20)	HER2-positive (n = 7)	TN basal-like (n = 17)	TN normal-like (n = 13)	*p*
Age, years								
Mean ± SD	11.4 ± 2.82	10.5 ± 2.1	11.4 ± 2.5	11.1 ± 3.2	12.4 ± 4.2	12 ± 3.0	11.9 ± 2.8	0.580
Age by group								
<8	9 (9.2%)	2 (13.3%)	1 (3.4%)	4 (20.0%)	1 (14.3%)	1 (6.2%)	0 (0.0%)	0.272
8–12	54 (55.1%)	10 (66.7%)	20 (69.0%)	8 (40.0%)	2 (28.6%)	9 (56.2%)	5 (45.5%)	
>12	35 (35.7%)	3 (20.0%)	8 (27.6%)	8 (40.0%)	4 (57.1%)	6 (37.5%)	6 (54.5%)	
Unknown[a]	4	0	1	0	0	1	2	
Breed								
Inbreed	82 (80.4%)	12 (80.0%)	24 (80.0%)	17 (85.0%)	7 (100%)	11 (64.7%)	11 (84.6%)	0.834
Siamese	9 (8.8%)	1 (6.7%)	2 (6.7%)	3 (15.0%)	0 (0.0%)	3 (17.6%)	0 (0.0%)	
Persian	7 (6.8%)	1 (6.7%)	2 (6.7%)	0 (0.00%)	0 (0.0%)	2 (15.4%)	2 (15.4%)	
NFC	3 (2.9%)	1 (6.7%)	1 (3.3%)	0 (0.0%)	0 (0.0%)	1 (5.9%)	0 (0.0%)	
Russian Blue	1 (1.0%)	0 (0.0%)	1 (3.3%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	
Spayed								
No	53 (53.0%)	10 (66.7%)	14 (46.7%)	8 (42.1%)	2 (28.6%)	9 (52.9%)	10 (83.3%)	0.125
Yes	47 (47.0%)	5 (33.3%)	16 (53.3%)	11 (57.9%)	5 (71.4%)	8 (47.1%)	2 (16.7%)	
Unknown[a]	2	0	0	1	0	0	1	
Progestogens								
No	33 (40.7%)	4 (33.3%)	12 (48.0%)	6 (42.9%)	2 (33.3%)	5 (35.7%)	4 (40.0%)	
Yes	48 (59.3%)	8 (66.7%)	13 (52.0%)	8 (57.1%)	4 (66.7%)	9 (64.3%)	6 (60.0%)	0.951
Unknown[a]	21	3	5	6	1	4	2	
Multiple T								
No	52 (51.0%)	9 (60.0%)	16 (53.3%)	10 (50.0%)	2 (28.6%)	8 (47.1%)	7 (53.8%)	0.833
Yes	50 (49.0%)	6 (40.0%)	14 (46.7%)	10 (50.0%)	5 (71.4%)	9 (46.2%)	6 (46.2%)	
LNS								
Negative	53 (57.0%)	9 (64.3%)	16 (61.5%)	8 (42.1%)	3 (50.0%)	9 (60.0%)	8 (61.5%)	0.773
Positive	40 (43.0%)	5 (35.7%)	10 (38.5%)	11 (57.9%)	3 (50.0%)	6 (40.0%)	5 (38.5%)	
Unknown[a]	9	1	4	1	1	2	0	
Stage								
I	22 (21.6%)	2 (13.3%)	6 (20.0%)	6 (30.0%)	1 (14.3%)	4 (23.5%)	3 (23.1%)	0.719
II	17 (16.7%)	5 (33.3%)	6 (20.0%)	1 (5.0%)	2 (28.6%)	2 (11.8%)	1 (7.7%)	
III	47 (46.1%)	6 (40.1%)	14 (46.7%)	10 (50.0%)	4 (57.1%)	6 (35.3%)	7 (53.8%)	
IV	16 (15.7%)	2 (13.3%)	4 (13.3%)	3 (15.0%)	0 (0.0%)	5 (29.4%)	2 (15.4%)	
Treatment								
None	9 (8.8%)	0 (0.0%)	1 (3.3%)	2 (10.0%)	0 (0.0%)	4 (23.5%)	2 (15.4%)	–
Mastectomy	93 (91.2%)	15 (100%)	29 (96.7%)	18 (90.0%)	7 (100%)	13 (76.5%)	11 (84.6%)	
Surgery								
Lumpectomy	12 (12.9%)	2 (13.3%)	2 (6.90%)	2 (11.1%)	3 (42.9%)	1 (7.7%)	2 (18.2%)	–
Unilateral	73 (78.5%)	12 (80.0%)	25 (86.2%)	13 (72.2%)	4 (57.1%)	10 (76.9%)	9 (81.8%)	
Bilateral	8 (8.6%)	1 (6.70%)	2 (6.90%)	3 (16.7%)	0 (0.0%)	2 (15.4%)	0 (0.0%)	
Laterality								
Left	48 (47.5%)	7 (46.7%)	12 (41.4%)	9 (45%)	4 (57.1%)	10 (58.8%)	6 (46.2%)	0.897
Right	53 (52.5%)	8 (53.3%)	17 (58.6%)	11 (55%)	3 (42.9%)	7 (41.2%)	7 (53.8%)	
Unknown[a]	1	0	1	0	0	0	0	

Abbreviations: LNS, Lymph node status; NFC, Norwegian forest cat; SD, standard deviation; T, tumor; TN, triple negative.
[a] Not considered in the calculation of the percentages neither in the statistical analysis.

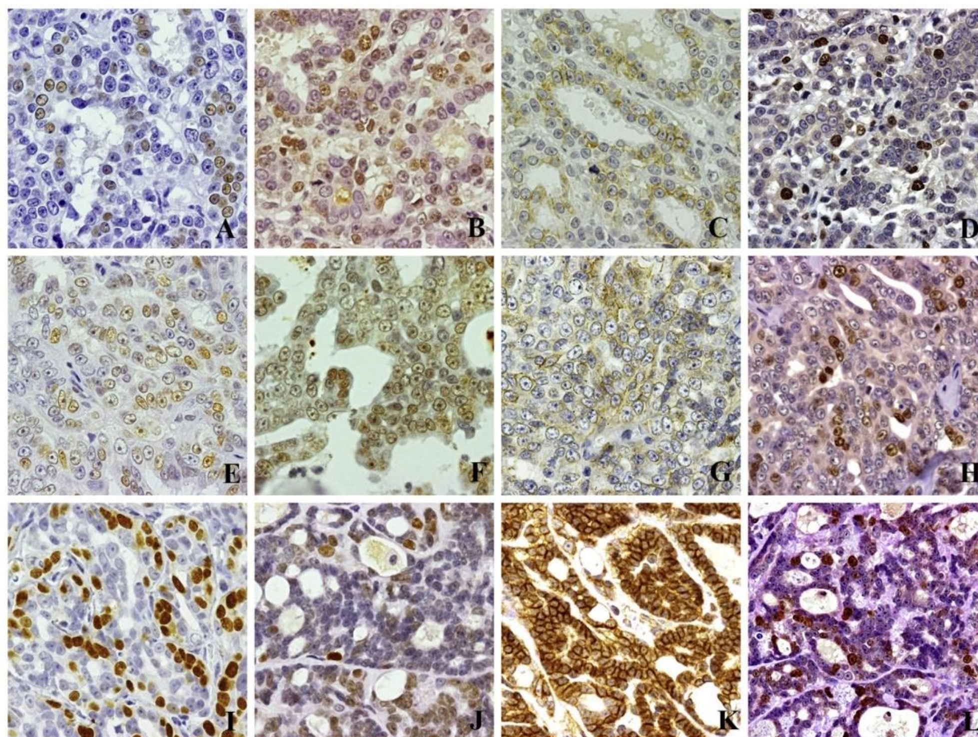

Fig. 1. Immunohistochemical expression of the different proteins studied in FMC. **(A–D) Luminal A subtype** in a feline tubulopapillary carcinoma with positive score for ER (A), PR (B), *f*HER2-negative status (C) and 9% Ki-67 index (D); **(E–H) Luminal B/HER2-negative subtype** in a feline tubulopapillary carcinoma presenting positive immunostaining for ER (E) and PR (F), negative score for *f*HER2 (G) and a high Ki-67 index of 21.3% (H); **(I–L) Luminal B/HER2-positive subtype** in a feline tubulopapillary carcinoma with overexpression of ER (I), PR (J), *f*HER2 (K) and with any Ki-67 index, which was estimated as 15.2% (L). Original magnification ×400 (Mayer's hematoxylin).

of disease, histopathological classification, malignancy grade, presence of necrotic areas, lymphatic invasion by neoplastic cells, lymphocytic infiltration and cutaneous ulceration. For this analysis, animals were divided in groups by age (<8 years; 8–12 years and >12 years), and tumor size (<2 cm; 2–3 cm; >3 cm), according to TNM classification [1]. For each significant association, the odds ratio (OR) was calculated with a confidence interval (CI) of 95%.

In survival analysis, OS time was calculated, in days, from the date of the initial diagnosis to the date of death/euthanasia due to neoplasia or the date of the last follow-up for the living queens (censored observations). Only the mammary carcinoma-related deaths were considered, while the deaths caused by other processes were censored data. The DFS time was measured, in days, from the date of the first surgery to the date of the first in loco regional or systemic relapse, or the death from cancer disease. Animals without relapses or lost during the follow-up were censored at the last follow-up. Animals that the moment of the relapse could not be determined by the clinician were excluded from this study. Survival curves were determined using the Kaplan–Meier method and differences between the molecular

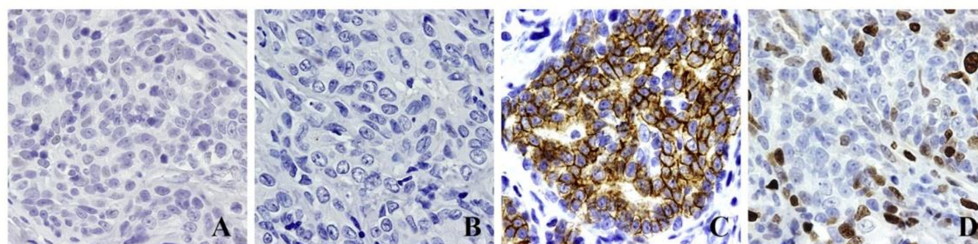

Fig. 2. Immunohistochemical expression of the different proteins studied in FMC. **HER2-positive subtype** in a solid mammary carcinoma. Tumor sections were negative for ER (A) and PR (B) but presented overexpression of *f*HER2 protein (C). The neoplastic cells showed a high Ki-67 proliferation index of 30.8% (D). Original magnification ×400 (Mayer's hematoxylin).

subtypes concerning survival and disease free survival time were investigated by the log-rank test.

For the animals that presented multiple mammary tumors, the lesion used in the statistical analysis was selected considering the size (the larger tumor) and the malignancy grade (the most malignant lesion), as these parameters have been already associated with poor prognosis in FMC [29,33].

Results

Clinicopathological features

The clinical features of the 102 queens enrolled in this study are summarized in Table 4. The luminal B/HER2-negative was the most common subtype (Fig. 1), with a frequency of 29.4% (30/102) followed by the luminal B/HER2-positive subtype 19.6%, (20/102). The triple negative basal-like (Fig. 3) was the third most common subtype with 16.7% (17/102), closely followed by luminal A (Fig. 1) with14.7% (15/102) and the triple negative normal-like subtype (Fig. 3) with 12.7% (13/102). Finally, HER2-positive subtype (Fig. 2) was the rarer, with a frequency of 6.9% (7/102).

The histopathological characteristics of the 229 mammary tumors, which were collected from the 102 female cats, are summarized in Table 5.

Associations between FMC molecular subtypes and clinicopathological features

No statistical differences were found between the molecular subtype and the clinical features (Table 4). Considering the histopathological characteristics (Table 5), tumor size ($p < 0.001$), malignancy grade ($p < 0.001$) and the presence of necrosis ($p = 0.003$) revealed significant associations between the molecular subtypes.

Concerning tumor size, the triple negative basal-like and the triple negative normal-like subtypes were significantly larger when compared with other cancer subtypes ($p < 0.001$). Furthermore, when mammary tumors were grouped according to the TNM staging system, differences between size classes were also statistical significant ($p = 0.024$), with the luminal A subtype associated with smaller tumors (OR 3.3; 95% CI 0.95–17.6), and the triple negative basal-like subtype associated with larger tumors (OR 2.59; 95% CI 1.12–5.84).

Luminal A subtype was associated with well-differentiated tumors (OR 3.5; 95% CI 1.12–9.18) contrasting with luminal B/HER2-positive (OR 2.6; 95% CI 1.04–6.33), triple negative basal-like (OR 6.5; 95% CI 1.00–272.6) and triple negative normal-like (OR 3.6; 95% CI 1.03–19.93) subtypes that were associated with poorly differentiated tumors (grade III).

Statistical analysis also revealed that triple negative basal-like (OR 3.1, 95% CI 1.26–8.71) and triple negative normal-like subtypes (OR 3.89, 95% CI 1.09–21.18) were positively associated with tumor necrosis, contrasting with luminal B/HER2-positive tumors (OR 2.1; 95% CI 1.09–4.06) that were negatively associated.

Survival analysis

Follow-up data were available for OS study in 90 queens. The number of tumor related deaths at the end of the study was 45 (50%) and the mean overall survival was 671.7 days (SD ± 63.4 days). There were no statistical differences in the mean survival time for each molecular subtype ($p = 0.226$), but the luminal A subtype showed a better outcome, with the highest survival time (mean OS = 943.6 days, SD ± 166.8). It was followed by the triple negative normal-like (mean OS = 725 days, SD ± 163.8), luminal B/HER2-negative (mean OS = 625.2 days, SD ± 97.2), luminal B/HER2-positive (mean OS = 568.3 days, SD ± 98.2) and HER2-positive subtype (mean OS = 432.3 days, SD ± 108.9). Finally, the triple negative basal-like subtype presented the lowest survival time, with a OS mean of 368.9 days (SD ± 83.3).

In the DFS study only 77 animals were considered. Relapse of the disease was recorded in 46 queens (59.7%, 46/77) and of these, 34 (73.9%, 34/46) presented loco regional relapse, while 12 animals (26.1%, 12/46) presented distant metastasis. The mean for disease free survival period was 464.9 days (SD ± 49.9). Although it was not statistically significant ($p = 0.065$, Fig. 2B), luminal A subtype presented the higher free disease survival time (mean 619.2 days, SD ± 73.6) followed by luminal B/HER2-positive (511.1 days, SD ± 113.6), luminal B/HER2-negative subtype (372.2 days, SD ± 61.5), triple negative basal-like subtype (322.6 days, SD ± 67),

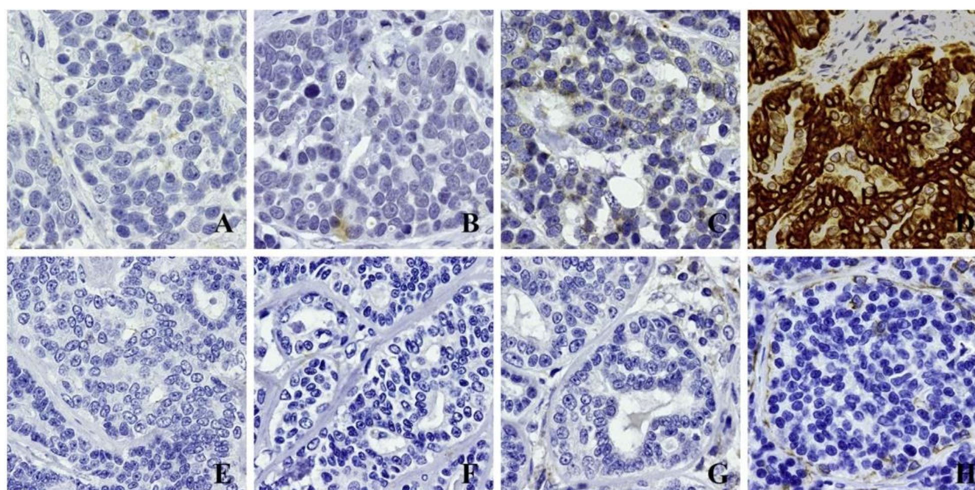

Fig. 3. Immunohistochemical expression of the different proteins studied in FMC. **Triple negative subtypes** in FMC. **Triple negative basal-like subtype** in a case of solid mammary carcinoma (A–D) being ER- (A), PR- (B) and *f*HER2- (C) and positive for CK 5/6 (D). **Triple negative normal-like subtype** in a case of tubulopapillary mammary carcinoma **(E–H)** being ER- (E), PR- (F), *f*HER2- (G) and CK 5/6- (H). Original magnification ×400 (Mayer's hematoxylin).

Table 5
FMC features and associations with the molecular subtype.

Histopathological features	Total (n = 229)	Luminal A (n = 37)	Luminal B/HER2− (n = 62)	Luminal B/HER2+ (n = 57)	HER2-positive (n = 11)	TN basal-like (n = 40)	TN normal-like (n = 22)	*p*
Localization								
M1	38 (17.0%)	2 (5.7%)	13 (21.7%)	11 (20.0%)	2 (18.2%)	6 (15.0%)	4 (18.2%)	0.264
M2	54 (24.2%)	8 (22.8%)	18 (30.0%)	11 (20.0%)	2 (18.2%)	13 (32.5%)	2 (9.1%)	
M3	74 (32.8%)	15 (42.9%)	18 (30.0%)	16 (29.1%)	3 (27.3%)	13 (32.5%)	9 (40.9%)	
M4	57 (26.0%)	10 (28.6%)	11 (18.3%)	17 (30.9%)	4 (36.3%)	8 (20.0%)	7 (31.8%)	
Unknown[a]	6	2	2	2	0	0	0	
Size, cm								
Mean ± SD	2.11 ± 1.59	1.59 ± 1.1	1.85 ± 1.29	1.84 ± 1.48	2.18 ± 1.6	2.86 ± 2.01	3.00 ± 1.74	<0.001
Size by groups								
<2	113 (49.8%)	22 (59.5%)	29 (46.8%)	34 (61.8%)	4 (36.3%)	16 (40.0%)	8 (36.4%)	0.024
2–3	68 (30.0%)	12 (32.4%)	23 (37.1%)	12 (21.8%)	5 (45.5%)	10 (25.0%)	6 (27.3%)	
>3	46 (20.2%)	3 (8.10%)	10 (16.3%)	9 (16.4%)	2 (18.2%)	14 (35.0%)	8 (36.4%)	
Unknown[a]	2	0	0	2	0	0	0	
HP classification								
"in situ" carcinoma	10 (4.4%)	2 (5.4%)	2 (3.2%)	5 (8.8%)	0 (0.0%)	0 (0.0%)	1 (4.5%)	0.064
TP carcinoma	135 (59.0%)	28 (75.7%)	36 (58.9%)	35 (61.4%)	4 (36.7%)	20 (50.0%)	12 (54.5%)	
Mucinous carcinoma	15 (6.6%)	0 (0.0%)	4 (6.5%)	0 (0.0%)	2 (18.2%)	4 (10.0%)	5 (22.7%)	
Cribriform carcinoma	34 (14.8%)	4 (10.8%)	9 (14.5%)	7 (12.3%)	2 (18.2%)	9 (22.5%)	3 (13.6%)	
Solid carcinoma	33 (14.4%)	2 (5.4%)	11 (17.7%)	9 (15.7%)	3 (27.3%)	7 (17.5%)	1 (4.5%)	
SC carcinoma	1 (0.4%)	0 (0.0%)	0 (0.0%)	1 (1.8%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	
Carcinosarcoma	1 (0.4%)	1 (2.7%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	
Malignancy grade								
I	28 (12.2%)	10 (27.0%)	4 (6.5%)	12 (21.1%)	0 (0.0%)	1 (2.5%)	1 (4.5%)	<0.001
II	51 (22.3%)	11 (29.7%)	15 (24.2%)	16 (28.0%)	1 (9.1%)	6 (15.0%)	2 (9.1%)	
III	150 (65.5%)	16 (43.3%)	43 (69.3%)	29 (50.9%)	10 (90.9%)	33 (82.5%)	19 (86.4%)	
Necrosis								
No	82 (35.8%)	18 (48.6%)	23 (37.0%)	28 (49.1%)	3 (27.3%)	7 (17.5%)	3 (13.6%)	0.003
Yes	147 (64.2%)	19 (51.4%)	39 (63.0%)	29 (50.9%)	8 (72.7%)	33 (82.5%)	19 (86.4%)	
Lymphatic invasion								
No	188 (82.1%)	36 (97.3%)	50 (80.6%)	44 (77.2%)	8 (72.7%)	31 (77.5%)	19 (86.4%)	0.138
Yes	41 (17.9%)	1 (2.7%)	12 (19.4%)	13 (22.8%)	3 (27.3%)	9 (22.5%)	3 (13.6%)	
LI								
No	84 (36.7%)	14 (37.8%)	25 (40.3%)	20 (35.1%)	2 (18.2%)	14 (35.0%)	9 (40.9%)	0.810
Yes	145 (63.3%)	23 (62.2%)	37 (59.7%)	37 (64.9%)	9 (81.8%)	26 (65.0%)	13 (59.1%)	
Ulceration								
No	204 (89.1%)	35 (94.6%)	57 (92.0%)	53 (93.0%)	9 (81.8%)	34 (85.0%)	16 (72.7%)	0.078
Yes	25 (10.9%)	2 (5.4%)	5 (8.0%)	4 (7.0%)	2 (18.2%)	6 (15.0%)	6 (27.3%)	
ER status								
Negative	148 (64.6%)	8 (21.6%)	39 (63.0%)	28 (49.1%)	11 (100%)	40 (100%)	22 (100%)	<0.001
Positive	81 (35.4%)	29 (78.4%)	23 (37.0%)	29 (50.9%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	
PR status								
Negative	130 (56.8%)	21 (56.8%)	15 (24.2%)	21 (36.8%)	11 (100%)	40 (100%)	22 (100%)	<0.001
Positive	99 (43.2%)	16 (43.2%)	47 (75.8%)	36 (63.2%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	
HER2 status								
Negative	161 (70.3%)	37 (100%)	62 (100%)	0 (0.0%)	0 (0.0%)	40 (100%)	22 (100%)	<0.001
Positive	68 (29.7%)	0 (0.0%)	0 (0.0%)	57 (100%)	11 (100%)	0 (0.0%)	0 (0.0%)	
Ck 5/6 status								
Negative	91 (39.9%)	14 (37.8%)	28 (45.9%)	22 (38.6%)	5 (45.5%)	0 (0.0%)	22 (100%)	<0.001
Positive	137 (60.1%)	23 (62.2%)	33 (54.1%)	35 (61.4%)	6 (54.5%)	40 (100%)	0 (0.0%)	
Unknown[a]	1	0	1	0	0	2		
Ki-67 index (%)								
Mean ± SD	23.5 ± 17.7	6.9 ± 3.9	25.4 ± 10.2	20.8 ± 17.7	34.5 ± 13.7	31.6 ± 23.2	32.3 ± 19.1	<0.001
Ki-67 by groups								
Low	83 (36.2%)	37 (100%)	0 (0.0%)	30 (52.6%)	0 (0.0%)	11 (27.5%)	5 (22.7%)	
High	146 (63.8%)	0 (0.0%)	62 (100%)	27 (47.4%)	11 (100%)	29 (72.5%)	17 (77.3%)	<0.001

[a] Not considered in the calculation of the percentages neither in the statistical analysis.; Abbreviations: HP, Histopathological; SC, Squamous cell; LI, Lymphocytic infiltration; SD, standard deviation; TP, Tubulopapillary.

triple negative normal-like (214.4 days, SD ± 42.7) and, finally, the HER2-positive subtype (203.4 days, SD ± 37.8).

Concordance of the molecular subtypes of primary tumors from the same animal

In 50 queens (49%, 50/102) multiple mammary masses were present at the initial diagnosis, ranging from 2 to 5 independent tumors: 24 presented 2 mammary masses (48%, 24/50), 14 had 3 tumors (28%, 14/50), 9 showed 4 tumors (18%, 9/50) and, finally, 3 had 5 tumors (6%, 3/50).

In 18 out of the 50 cats (36%) the molecular classification was equal in the different primary tumors. The remaining 32 animals (64%, 32/50) presented discordant classifications between the primary tumors. The most common association of phenotypes in the same animal was the luminal B/HER2-negative and luminal B/HER2-positive that was found in 5 cases (16%, 5/32).

Discussion

The six molecular subtypes of mammary tumors were identified in the feline population studied with the luminal B/HER2-negative being the most common subtype (29.4%) followed by luminal B/

HER2-positive (19.6%) and by triple negative basal-like subtypes (16.7%). These results differ from the published data [37], according to which the triple negative basal-like subtype was the most prevalent, but are concordant with Brunetti et al. (2013) that identified the luminal B subgroup as the most common in FMC.

In human breast cancer, the luminal A subtype is the most common [12,15,47,48], contrasting with HER2-positive and triple negative subtypes (basal-like and normal-like) that usually present the lowest frequencies [12,13,47].

In our study, luminal A subtype was significantly associated to a lower size and a low histological grade (Grade I), similarly to what is described in human breast cancer and in canine mammary carcinomas, in which this subtype has been associated with low histological grade, less invasive and less proliferative tumors. Also, the luminal A subtype presented the longest survival time (943.6 days) and disease free survival time (619.2 days), supporting that these tumors could be associated to a more favorable outcome in cats, like it is described for humans [15,49].

Unlike others feline studies [35,37], we have divided the luminal B type into two subtypes, luminal B/HER2-negative and luminal B/HER2-positive, as is recommended by St. Gallen panel.

Despite the similar features among the two subtypes, the luminal B/HER2-positive was significantly associated to malignancy grade, presenting a 2.3 times higher odds (OR = 2.3) of being an undifferentiated tumor (Grade III), when compared to the other subtypes.

Moreover, the luminal B/HER2-positive subtype was associated with absence of necrotic areas ($p = 0.003$, OR = 2.1), contrasting with triple negative subtypes (normal and basal-like), that were significantly associated with the presence of necrosis, a characteristic usually related with more aggressive tumors, corroborating that, similarly with what is described for humans, luminal B tumors present less aggressive features, when compared with the non-luminal subtypes [50].

Triple negative basal-like and normal-like subtypes were also significantly correlated to larger tumors with higher malignancy grades (Grade III), once again resembling what is described for humans. In fact, triple negative and HER2-positive subtypes are usually associated to a worse prognosis, presenting lower survival time in woman patients [15,22,50]. The triple negative type is generally divided in the normal-like and basal-like subtype, on basis of the expression of basal markers, like cytokeratins 5/6 [13,50–52]. According to St. Gallen panel, approximately 80% of the triple negatives tumors have an intrinsic basal-like subtype, making the distinction between the two subtypes less significant in the human disease [14]. In our study, only 57% of the triple negative tumors were basal-like, supporting the maintenance of the distinction of the two subtypes, as was performed by other veterinary studies [35,37,49,53]. The distinction between the two subtypes proved to be relevant, as they presented very distinct survival times in cats: the animals that presented triple negative basal-like tumors display the shorter OS (368.9 days), while the cats with normal-like tumors present the second larger OS (725 days).

The HER2-positive subtype showed the second lowest survival (432.3 days) and the lowest DFS (203.4 days) time. This subtype also demonstrated, together with the triple negative, higher proliferation capability, when compared with the luminal types (31.6%–34.5% in the non-luminal subtypes *versus* 6.9%–25.4% in the luminal subtypes).

One of the major difficulties in our study was the high percentage of animals with multiple mammary masses at the time of the diagnosis. Unlike humans, where synchronous bilateral breast carcinomas are uncommon, the presence of multiple masses (usually in the same mammary chain) at the moment of the diagnosis is very common in cats, with previous studies pointing to a prevalence higher than half of the cases [1,54]. This represents a serious difficulty, either for the interpretation of the results and comprehension of tumor disease behavior, as for disease staging, clinical and therapeutic management.

One of the objectives of our study was to evaluate if the different primary mammary lesions in the same female cat presented the same molecular phenotype. The implications of the high heterogeneity of the mammary tumors require more studies, in order to improve the management of the disease and to increase the basic knowledge of cancer behavior.

Conclusion

In this study, we demonstrated the molecular heterogeneity of feline mammary carcinomas, and we were able to identify and characterize 6 different molecular subtypes, presenting several similarities with the human breast cancer disease. In fact, clinicopathological characterization of the different subtypes was similar to what is described for woman, what lead us to believe that FMC would be a suitable natural model for the study of human breast cancer disease, especially for luminal B/HER2-negative, luminal B/HER2-positive and triple negative basal-like subtypes.

For veterinary clinical practice, this classification open perspectives for better prognostic evaluation and towards the development of new and more specific treatments, directed against target proteins, which will improve the overall and the disease free survival of the feline patients with mammary cancer.

Conflict of interest statement

The authors declare that they have no competing interests.

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ANNEX IV – St Gallen molecular subtypes in feline mammary carcinoma and paired metastases – disease progression and clinical implications from a 3-year follow-up study

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ORIGINAL ARTICLE

St Gallen molecular subtypes in feline mammary carcinoma and paired metastases—disease progression and clinical implications from a 3-year follow-up study

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Abstract Considering that scarce data are available on disease progression of feline mammary carcinoma (FMC), this study aimed to analyze the clinical, pathological, and immunophenotypic features collected from 61 queens with FMC and to compare the concordance ratios of the expression levels of five molecular markers (ER, PR, *f*HER2, CK5/6, and Ki-67) between primary tumors (PT) and metastatic lesions. The results showed that cats with luminal A mammary carcinomas (MC) had higher overall survival (924.6 days, $p=0.001$) and longer disease-free period (385.4 days, $p=0.005$) compared to the ones with other MC subtypes. In fact, queens with triple negative/basal-like MC showed the lowest survival (mean 156.2 days) and the shortest disease-free survival (mean 28 days) among the molecular subtypes of MC. The lung was the organ most frequently affected by metastases, and animals with lung and/or pleural metastases were more likely to display metastases at three or more locations ($p=0.039$). A large heterogeneity in protein expression levels was found between PT and paired metastases, with both estrogen and progesterone receptors more likely to be downregulated in metastases. Paired metastases frequently had higher Ki-67 index than PT, whereas *f*HER2 overexpression was seen in 46 samples (30 %) and CK5/6 expression was found in 50.7 % of metastases (36/71). Results also revealed that disease progression leads to a high percentage of triple negative/basal-like metastases (9/23; 39.1 %) associated with the absence of luminal A subtype in distant metastases (0/23). This study highlights the prognostic importance of immunophenotyping of MC in cats, although the modified protein expression identified in metastases contributes to justify why possible targeted therapies may fail in some animals with metastatic disease. Altogether, the results obtained also demonstrate that FMC can be used as a model to study human breast cancer.

Keywords Feline mammary carcinoma · Metastatic disease · Molecular classification · Prognostic biomarkers

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Introduction

Feline mammary tumors (FMT) are the third most common tumor in cats, representing approximately 17 % of all neoplasms in this species [1]. Feline mammary carcinomas (FMC) are by far the most representative lesion within this group of tumors, showing a high metastatic potential [1, 2]. Up to now, very limited data are available regarding disease progression in FMC, although metastatic lesions are known to be the main cause of death, representing a major clinical and therapeutic challenge [3–5].

In breast cancer patients, recent studies have shown contradictory results, with some authors reporting a similar gene expression profile between the primary tumor and the paired metastases [3, 6], while the majority demonstrates a marked heterogeneity of the molecular signatures between primary and metastatic lesions [7–10]. These data are relevant to optimize patients' management since prescribed targeted therapies consider the breast tumor subtype [11–13].

In cats, recent studies showed that several biomarkers have prognostic value in mammary carcinoma [14–21], although

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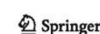

the immunophenotyping of mammary tumors failed to add significant prognostic value [22–24].

In this study, clinical information collected from a study population of 61 queens with mammary carcinoma was evaluated, and the gene expression profiles of primary mammary carcinomas were compared with the ones obtained in local, regional, and distant metastases. Finally, correlation studies were performed between the clinicopathological features and the expression of biomarkers and also between the clinicopathological features and the molecular subtypes of the primary tumor/metastases.

Material and methods

Animal patients

Owners gave permission to collect samples from their pets and to use the animal's clinical data. Tumor samples were collected in accordance with the EU Directive 2010/63/EU and research was approved by the ethics committee of the Faculty of Veterinary Medicine (FVM), University of Lisbon (ULisboa).

The cohort study enrolled 61 queens with spontaneous mammary carcinomas, presented at the Teaching Hospital of the FVM-ULisboa from June 2011 to May 2014. Only cats that had one form of disease (local relapse, regional metastases, or distant metastases), at the time of the diagnosis or during the follow-up period, were included in the study. During the follow-up, animals were examined at least once every 6 months. Although 126 cats were initially selected for this study, 65 were excluded because nine showed benign mammary tumors, 31 with mammary carcinomas lacked any form of disease progression, 21 had failed the clinical periodic examinations, and four animals died from other diseases.

In order to maximize survival, a complete surgical resection of the primary tumor with tumor-free margins of 2 cm was performed in all cats that underwent to surgery (51/61, 83.6 %). The remaining ten cats did not receive any treatment once they already presented distant metastases. From the selected animals (61/126), 21 (34.4 %) had local relapse during the follow-up period, and 48 (78.7 %) showed lymph node involvement at the time of the first diagnosis. A complete necropsy was performed in 23 (37.7 %) animals in the Anatomical Pathology Laboratory (FMV-ULisboa). Tissue samples from the primary tumor and, whenever possible, from metastases (regional lymph nodes and distant organs) were fixed in 10 % buffered formalin for 24 to 48 h and embedded in paraffin blocks. The following clinical data were recorded when available: age, breed, reproductive status (intact vs. spayed), administration of progestogens, number and location of tumors, tumor stage according to TNM system [1], prescribed treatment (none, mastectomy, mastectomy combined with chemotherapy), overall survival (OS), and disease-free survival (DFS) times.

Histopathological and immunohistochemical analysis

All tumor lesions were classified according to the World Health Organization classification system, and malignancy grade was determined by the use of two different systems: the Elston and Ellis scoring system and a novel grading system for evaluation of invasive FMC published by Mills et al. (2014), which considers lymphovascular invasion, nuclear form, and mitotic count [25–27]. The presence of necrotic areas, lymphatic vessel invasion by tumor cells, lymphocytic infiltration, and skin ulceration were also evaluated.

All primary tumors and paired metastases (local, regional, and/or distant) were submitted to immunohistochemistry (IHC) using the Novocastra Novolink Polymer Detection System (Leica Biosystems) to detect estrogen (ER) and progesterone receptors (PR), feline homologue of human epidermal growth factor receptor type 2 (*f*HER2), cytokeratin 5/6 (CK5/6) and Ki-67, as summarized in Table 1 and as previously described [14, 22, 23].

Briefly, the anti-ER antibody (clone 6 F11, 1:100 dilution; Thermo Scientific, Rockford, USA), the anti-HER2 antibody (clone CB11, 1:200 dilution; Invitrogen, Carlsbad, USA), and the anti-Ki-67 polyclonal antibody (1:500 dilution; Thermo Scientific) were applied to the samples after antigen retrieval in sodium citrate buffer (0.01 M $NaCH_3COO$, pH 6.0), in a pressure cooker for 2 min at 2 atm. For ER and *f*HER2 detection, the antibodies were incubated overnight at 4 °C, whereas a 60-min incubation period, at room temperature, was performed for Ki-67. PR staining was conducted with the ready-to-use 1E2 antibody (Ventana, Tucson, USA), incubated overnight at 4 °C, after antigen retrieval in 0.01 M sodium citrate solution (pH 6.0) in a water bath at 95 °C, for 60 min. The anti-CK 5/6 antibody (clone D5/16 B4, ready to use; Ventana) was applied overnight, at 4 °C after a heat-induced antigen retrieval procedure (microwave for 15 min) with Tris-EDTA buffer (pH 9.0).

Biomarker expression from IHC assays was scored by two independent observers who were blinded to the clinicopathological features of the samples and to prognostic outcome of the queens. Discordant interpretations were further debated and settled using a multiviewer microscope.

Tissue samples were considered positive for ER or PR when the Allred score was equal or higher than 3 [23, 28, 29]. Quantitative immunohistochemical evaluation of *f*HER2 expression was performed by using the HercepTest scoring system [30] and samples with a score of 2 or 3 were considered positive [15, 17, 20, 31–33]. Tissue samples were considered to be positive for CK5/6 whenever more than 1 % of the tumor cells showed positive staining in the cytosol and/or in the cytoplasmic membrane [22]. The Ki-67 index was

Table 1 Primary antibodies and immunohistochemical protocols used for ER, PR, *f*HER2, CK5/6, and Ki-67 detection

Primary antibody	Dilution	Incubation	Antigen retrieval method
Mouse anti-ER-α, clone 6F11[a]	1:100	O/N, 4 °C	Pressure cooker (2 atm, 2′) with sodium citrate buffer
Rabbit anti-PR, clone 1E2[b]	RTU	O/N, 4 °C	Water bath (95 °C, 60′) with sodium citrate buffer
Mouse anti-HER2, clone CB11[c]	1:200	O/N, 4 °C	Pressure cooker (2 atm, 2′) with sodium citrate buffer
Mouse anti-CK5/6, clone D5/16 B4[a]	RTU	O/N, 4 °C	Microwave (900 W, 15′) with Tris-EDTA buffer (pH 9.0)
Rabbit anti-Ki-67, polyclonal[a]	1:500	60 min, RT	Pressure cooker (2 atm, 2′) with sodium citrate buffer

O/N overnight, *RT* room temperature, *RTU* ready-to-use

[a] Thermo Scientific, Rockford, USA

[b] Ventana, Tucson, USA

[c] Invitrogen, Carlsbad, USA

estimated by dividing the number of tumor cells with positive nuclear staining by the total number of tumor cells analyzed (at least 1000 cells), and samples were considered highly proliferative when at least 14 % of the tumor cells stained positive for Ki-67 [34].

All microscope procedures were performed using an Olympus BX51 light microscope (Pennsylvania, USA) equipped with an Olympus DP25 camera for image capturing. The images were analyzed using Image J (Open Source Software, version 1.46r, National Institutes of Health, Bethesda, USA).

Definition of the molecular subgroups

Feline mammary carcinomas were grouped in six immunophenotypes using the following recommendations of the St. Gallen International Expert Consensus panel [35]: luminal A (ER+ and/or PR+, *f*HER2−, any CK5/6 status, and low Ki-67 index), luminal B/HER2-negative (ER+ and/or PR+, *f*HER2−, any CK5/6 status, and high Ki-67 index); luminal B/HER2-positive (ER+ and/or PR+, *f*HER2+, any CK5/6 status, and any Ki-67 index); HER2-positive (ER−, PR−, *f*HER2+, any CK5/6 status, and any Ki-67 index); triple negative/basal-like (ER−, PR−, *f*HER2−, CK5/6+, and any Ki-67 index) and triple negative normal-like (ER−, PR−, *f*HER2−, CK5/6-, and any Ki-67 index).

Statistical analysis

Statistical analysis was performed using SPSS 20.0 for Windows (SPSS Incorporation, IBM) and p values less than 0.05 ($p<0.05$) were considered to be statistically significant.

To analyze continuous variables, cases were divided in the following groups: <10 years and ≥10 years; tumors of <2 cm; 2 up to 3 cm; >3 cm; tumors with a Ki-67 index ≥14 % or of less than 14 %. Local relapse (LR) was defined as local relapse in the homolateral mammary chain or in the ipsilateral glands. Regional metastases (RM) were defined as presence of tumor cells in the regional lymph nodes (axillary or inguinal), and distant metastases (DM) defined whenever tumor tissue was present in any organ apart from the regional lymph nodes and the mammary glands.

OS time was calculated, in days, from the date of the initial diagnosis to the date of death/euthanasia due to neoplasia or the date of the last follow-up for the living queens (censored observations). DFS time was also estimated in days, from the date of the first surgery to the date of diagnosis of LR/RM/DM, or to the date of death/euthanasia due to cancer disease progression. Survival analysis was performed only with the animals that underwent mastectomy, while animals without any treatment or submitted to chemotherapy were excluded from this analysis. Additionally, cats that died from other diseases and animals without relapse or lost during the follow-up were also censored. Survival analysis was performed using Kaplan-Meier curves, and differences in the OS and the DFS were investigated by the log-rank test. Multivariate analysis using the Cox proportional hazard model was performed to determine the way in which OS and DFS were affected by the other co-variables. The hazard ratio (HR) was calculated with a 95 % confidence interval (95 % CI).

Associations between the different clinicopathological features were analyzed using the Fisher's exact test. Finally, the concordance of ER/PR/*f*HER2/CK5/6 status and Ki-67 index was compared between the primary tumor (PT) and the LR, between the PT and the RM, between the PT and the DM. In order to ensure the consistency of our results, the statistical analysis of the relapse lesions, regional and distant metastases, as well as the concordance determination were performed on cats that underwent mastectomy only.

Results

Animal population

A total of 61 queens with a mean age of 11.8±0.34 years were enrolled in this prospective study, and their clinicopathological features are summarized in Tables 2 and 3. Among these

Table 2 Clinical features of the cats diagnosed with mammary carcinoma (n=61)

Features	Number of animals (%)
Age, mean±SD (years)	11.8±0.34
<10 years	12 (20.7 %)
≥10 years	46 (79.3 %)
Unknown[1]	3
Breed	
Undetermined	44 (80.3 %)
Siamese	5 (8.2 %)
Norwegian Forest Cat	4 (6.6 %)
Persian	2 (3.3 %)
Blue Russian	1 (1.6 %)
Spayed	
No	27 (45.8 %)
Yes	32 (54.2 %)
Unknown[a]	2
Progestogens	
No	19 (44.2 %)
Yes	24 (55.8 %)
Unknown[a]	18
Treatment	
None	10 (16.4 %)
Mastectomy	43 (70.5 %)
Mastectomy plus chemotherapy	8 (13.1 %)
Multiple mammary tumors	
No	28 (45.9 %)
Yes	33 (54.1 %)
Lymph node status	
Negative	12 (21.1 %)
Positive	45 (78.9 %)
Unknown[1]	4
Disease stage (TMN)	
I	5 (8.2 %)
II	4 (6.6 %)
III	38 (62.3 %)
IV	14 (23.0 %)
Tumor location	
M1	9 (15.0 %)
M2	10 (16.7 %)
M3	21 (35.0 %)
M4	20 (33.3 %)
Unknown[a]	1

[a] Not considered in the calculation of the percentages

animals, 21 (34.4 %) showed a local relapse, 48 (78.7 %) lymph node metastases, and 23 (37.7 %) had distant metastases. Fifty-one animals (83.6 %) were submitted to mastectomy, whereas the remaining ten (16.4 %) did not receive this procedure. Additionally, eight were treated with anthracycline-based chemotherapy after mastectomy (1 mg/kg of doxorubicin, intravenously, every 3 weeks for a maximum of five treatments or until the cat developed progressive disease or concurrent illness).

At the moment of the first diagnosis, most of the mammary carcinomas were classified as stage III (62.3 %, 38/61) or IV (23 %, 14/61), and some as stage I (8.2 %, 5/61) or stage II (6.6 %, 4/61). The mean PT size was 2.9 cm±0.34, whereas LR lesions had a mean size of 1.86 cm (SD=0.34 cm). Primary tumors and LR lesions were mostly classified as tubulopapillary carcinomas (65.6 and 42.9 %, respectively), and were poorly differentiated (88.5 and 61.9 %, respectively). According to the Elston & Ellis classification system [25], most of the primary tumors were poorly differentiated (88.5 %, 54/61), with only a small percentage being moderately differentiated (9.8 %, 6/61) or well differentiated (1.6 %, 1/61). Using the novel grading system for evaluation of invasive FMC [27], the results obtained were quite similar, with most of the primary tumors showing high grade malignancy (57.4 %, 35/61), followed by intermediate grade (34.4 %, 21/61) and low grade MC (8.2 %, 5/61). Concerning local relapses, we found a decrease in the percentage of grade III carcinomas (61.9 %, 13/21, using the Elston & Ellis classification, and 42.8 %, 9/21, using the novel grading system) and an increase of grade I carcinomas (14.3 %, 3/61 or 28.6 %, 6/21, using the Elston & Ellis or the novel classification, respectively).

Distribution pattern of subtypes and metastases

Both luminal B/HER2-negative (Fig. 1, a2–e2) and luminal B/HER2-positive (Fig. 1, a3–e3) were the most frequent subtypes in primary and relapse mammary carcinomas, although the triple negative/basal-like subtype (Fig. 1, a5–e5) was the most common in DM (Table 3) Fig. 2.

Ninety-three distant metastases were identified in 13 different locations (Table 4) and, interestingly, cats with lung and pleural metastases were more prone to having metastatic lesions in three or more sites (p=0.039). The lung and/or pleura (87 %), non-regional lymph nodes (70 %), and liver (30.4 %) were the most affected organs. Of the cats with metastatic disease, 34.8 % (8/23) had one or two metastatic sites, while 43.5 % (10/23) had three to five metastatic sites, and 21.7 % (5/23) had six to eight sites.

No association was found between the features of the primary tumor (histological type, malignancy grade, presence of necrotic areas, presence of lymphatic invasion by neoplastic cells, presence of lymphocytic infiltration, skin ulceration, biomarker status, and molecular classification) and the presence of lung metastases or the number of metastases per animal.

Survival analysis

Follow-up data concerning OS time was available for only 59 cats (559.7±65.1 days), since two had died from other diseases and were not considered for the survival analysis.

Table 3 Tumor features found in the studied cats

Features	Number of animals (%)			
	PT[8] (n=61)	LR[9] (n=21)	RM[10] (n=48)	DM[11] (n=23)
Tumor size, mean±SD[1] (cm)	2.90±0.21	1.86±0.34	–	–
<2 cm	14 (23.0 %)	12 (57.1 %)		
2–3 cm	24 (39.3 %)	4 (19.0 %)		
>3 cm	23 (37.7 %)	5 (23.9 %)		
HP[2] classification			–	–
Tubulopapillary carcinoma	40 (65.6 %)	9 (42.9 %)		
Solid carcinoma	8 (13.1 %)	3 (14.3 %)		
Cribriform carcinoma	8 (13.1 %)	5 (23.8 %)		
Mucinous carcinoma	5 (8.2 %)	0 (0.0 %)		
"in situ" carcinoma	0 (0.0 %)	3 (14.3 %)		
Squamous cell carcinoma	0 (0.0 %)	1 (4.8 %)		
MG[3] (EE[4] system)			–	–
I	1 (1.6 %)	3 (14.3 %)		
II	6 (9.8 %)	5 (23.8 %)		
III	54 (88.5 %)	13 (61.9 %)		
MG[3] (Mills et al. [27] system)			–	–
I	5 (8.2 %)	6 (28.6 %)		
II	21 (34.4 %)	6 (28.6 %)		
III	35 (57.4 %)	9 (42.8 %)		
Necrosis			–	–
No	14 (23.0 %)	7 (33.3 %)		
Yes	47 (77.0 %)	14 (66.7 %)		
Lymphatic invasion			–	–
No	45 (73.8 %)	17 (81.0 %)		
Yes	16 (26.2 %)	4 (19.0 %)		
Lymphocytic infiltration			–	–
No	11 (18.0 %)	10 (47.6 %)		
Yes	50 (82.0 %)	11 (52.4 %)		
Ulceration			–	–
No	51 (83.6 %)	21 (100.0 %)		
Yes	10 (16.4 %)	0 (0.0 %)		
ER status				
Negative	48 (78.7 %)	11 (52.4 %)	42 (87.5 %)	22 (95.7 %)
Positive	13 (21.3 %)	10 (47.6 %)	6 (12.5 %)	1 (4.3 %)
PR status				
Negative	29 (47.5 %)	10 (47.6 %)	24 (50.0 %)	16 (69.6 %)
Positive	32 (52.5 %)	11 (52.4 %)	24 (50.0 %)	7 (30.4 %)
*f*HER2 status				
Negative	42 (68.9 %)	12 (57.1 %)	36 (75.0 %)	17 (73.9 %)
Positive	19 (31.1 %)	9 (42.9 %)	12 (25.0 %)	6 (26.1 %)
CK5/6 status				
Negative	31 (51.7 %)	11 (52.4 %)	22 (45.8 %)	13 (56.5 %)
Positive	29 (48.3 %)	10 (47.6 %)	26 (54.2 %)	10 (43.5 %)
Ki-67 index, mean±SD[1] (%)	26.7±2.2	22.9±2.9	32.2±2.6	36.0±3.9
Low	14 (23.0 %)	6 (28.6 %)	8 (16.7 %)	2 (8.7 %)
High	47 (77.0 %)	15 (71.4 %)	40 (83.3 %)	21 (91.3 %)
Molecular Classification				
Luminal A	8 (13.1 %)	2 (9.5 %)	4 (8.3 %)	0 (0.0 %)
Luminal B/HER2-[5]	18 (29.5 %)	8 (38.1 %)	14 (29.2 %)	2 (8.7 %)
Luminal B/HER2+[6]	14 (23.0 %)	7 (33.3 %)	10 (20.8 %)	6 (26.1 %)
HER2-positive	4 (6.6 %)	2 (9.5 %)	3 (6.3 %)	0 (0.0 %)
TN[7] basal-like	10 (16.4 %)	1 (4.8 %)	11 (22.9 %)	9 (39.1 %)
TN[7] normal-like	7 (11.5 %)	1 (4.8 %)	6 (12.5 %)	6 (26.1 %)

SD standard deviation, *HP* histopathological, *MG* malignancy grade, *EE* Elston & Ellis [25], *Luminal/HER2−* Luminal B/HER2 negative, *Luminal/HER2+* Luminal B/HER2 positive, *TN* triple negative, *PT* primary tumor, *LR* local relapse, *RM* regional metastases, *DM* distant metastases

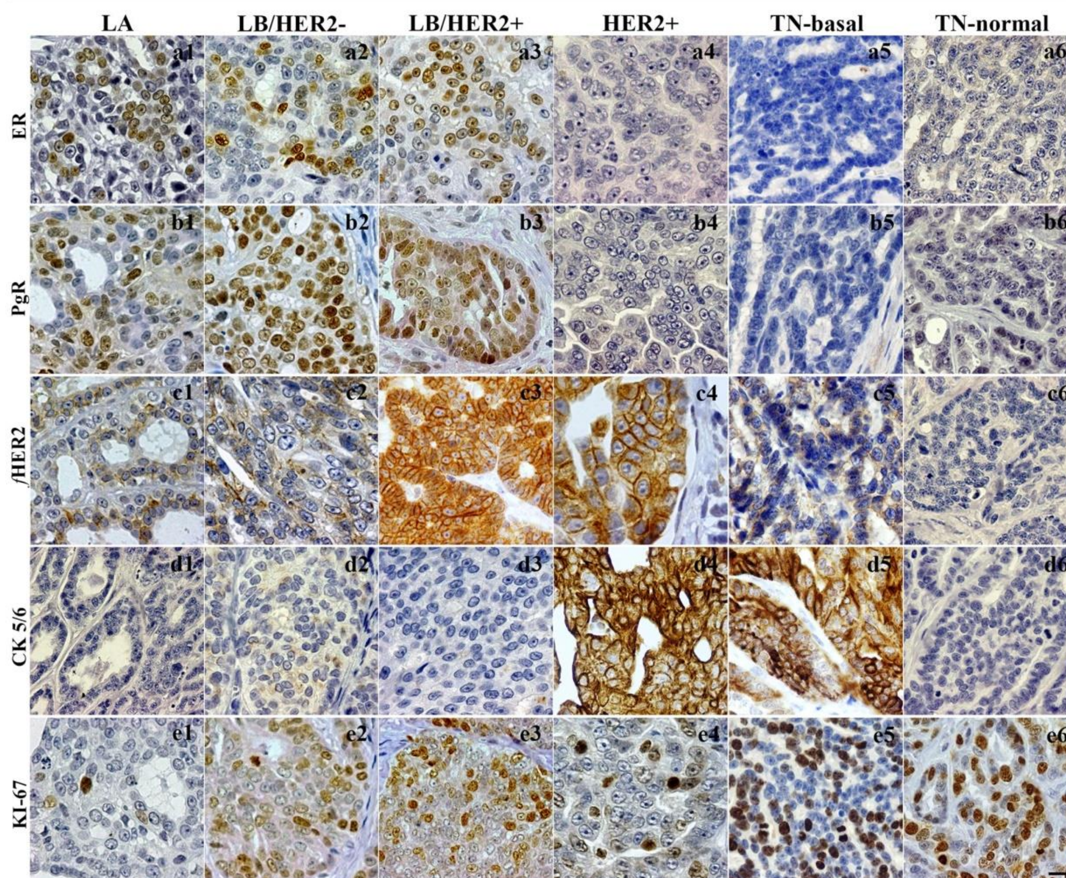

Fig. 1 Immunohistochemical classification of FMC into six subtypes on the basis of their ER, PR, *f*HER2, CK5/6, and Ki-67 expression. Luminal A FMC were defined as ER+ (a1) and/or PR+ (b1), *f*HER2- (c1), CK5/6- (d1) and a Ki-67 index of <14 % (e1). Luminal B/HER2-negative FMC were ER+ (a2) and/or PR+ (b2), *f*HER2- (c2), CK5/6- (d2) and a Ki-67 index of 14 % or greater (e2). FMC that were ER+ (a3) and/or PR+ (b3), *f*HER2+ (c3), CK5/6- (d3) and showed a Ki-67 index of ≥14 % (e3) were classified as luminal B/HER2-positive tumors. HER2-positive mammary carcinomas were ER- (a4), PR- (b4), *f*HER2+ (c4) and CK5/6+(d4), showing a Ki-67 index <14 % (e4). Triple negative/basal-like mammary carcinomas were ER- (a5), PR- (b5), *f*HER2- (c5), CK5/6+ (d5) and a Ki-67 index of ≥14 % (e5). Triple negative normal-like mammary carcinomas showed low expression of ER (a6), PR (b6), *f*HER2 (c6), and CK5/6 (d6) and a Ki-67 index equal or higher than 14 % (e6). *Scale bar*=20 μM

During the follow-up period, 40 animals were euthanized due to disease progression and despite the prescribed therapy (392 ±83.2 days). When the animals were divided according to the treatment prescribed, a significant difference in OS was found ($p<0.0001$) when comparing the animals without treatment (mean OS 179.9±82.5 days) versus those submitted only to mastectomy (559.9±76.1 days) or those submitted to surgery and chemotherapy (949.5±143.9 days).

Cats diagnosed with triple negative/basal-like or HER2-positive MC showed a significantly shorter survival time, when compared to other subtypes (156.2±70.9 days; 200.3± 93.1 days, respectively; $p=0.001$) (Fig. a2). Furthermore, animals with luminal A carcinomas showed the best survival (924.6±200.9 days), followed by queens with triple negative normal-like MC (725.3±276.5 days), luminal B/HER2-negative mammary carcinomas (448.1±71.5 days), and animals with luminal B/HER2-positive mammary tumors (447.1 ±61.4 days). In addition, cats with negative ER and/or positive CK5/6 status and/or high Ki-67 index (≥14 %) were significantly associated with lower OS ($p=0.04$, $p=0.022$, $p=0.011$, respectively; supplementary Table 1).

The DFS ranged from 222.3 to 284.9 days with a mean value of 253.6 days ($n=48$) and a median of 243±30 days. For the DFS study only the group of animals subjected to

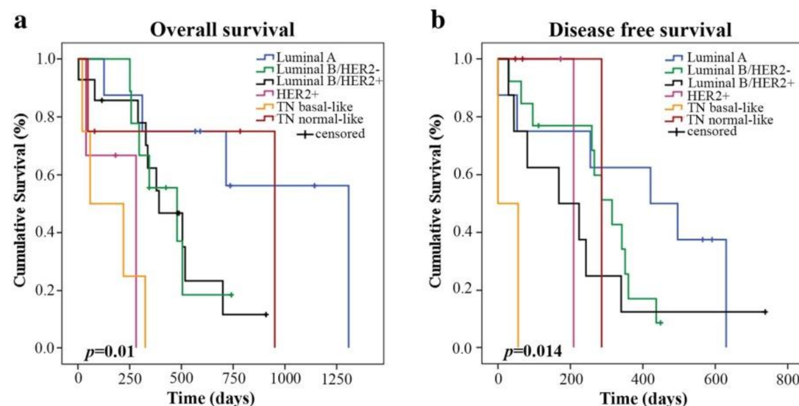

Fig. 2 Kaplan-Meier curves illustrating the OS (**a**) and DFS (**b**) of cats subjected to mastectomy (without chemotherapy) stratified according to the mammary tumor subtype. Cats with luminal A mammary carcinomas showed the longer OS (median 1308 days) and DFS period (median 421 days). In contrast, cats with triple negative/basal-like and HER2-positive mammary carcinomas experienced the shortest survival times (median 59 and 281 days, respectively), and the shortest DFS period (median 28 and 209 days, respectively). Cats with luminal B/HER2-positive mammary carcinomas showed intermediate survival times (median of 479 days), being followed by cats with luminal B/HER2-negative mammary carcinomas (median of 392 days) and by cats with triple negative/normal-like tumors (median of 951 days). Cats with luminal B/HER2-negative and triple negative normal-like mammary carcinomas showed the second longest DFS (315 and 286 days, respectively), followed by cats with HER2-positive carcinomas (median of 168 days)

surgery (without chemotherapy) was considered, in order to ensure sample homogeneity. The univariate analysis showed that cats with triple negative/basal-like MC had the shortest DFS (28±28 days, $p=0.014$, Fig. b2), followed by the group of cats with HER2-positive mammary carcinomas (209±25.5 days), the group with luminal B/HER2-positive tumors (233.4±76.2 days), the luminal B/HER2-negative group (276.6±36.5 days), the triple negative normal-like group (286±132 days) and, finally, by the group of cats with luminal A tumors (389.4±93.8 days).

Multivariable analysis

All variables that showed association in the univariate survival analysis ($p<0.05$) were included in the multivariable analysis (breed, number of primary tumors, stage, tumor size, malignancy grade using the Mills' score system, necrosis, lymphatic invasion, lymphocytic invasion, skin ulceration, ER status, CK 5/6 status, and Ki-67 index levels). Cox regression confirmed that molecular classification is an independent indicator of clinical outcome ($p=0.009$, Table 5) for OS but not for DFS ($p=0.398$, Table 6).

This multivariate analysis confirmed that a queen presenting a HER2-positive or a triple negative/basal-like mammary carcinoma has a higher risk of dying when compared to the other molecular subtypes (HR 438.4 [95 % CI, 14–13681.7] and HR 210.9 [95 % CI, 4.8-9352.3], respectively). In addition, the multivariate cox regression hazard analysis also showed that Ki-67 index levels ($p=0.035$) and the tumor size ($p=0.001$) were independent prognostic factors related to OS, with animals that had tumors with high Ki-67 index values or large size having a greater risk of dying from cancer disease (HR=34.6 [95 % CI, 1.3-926.5] and HR=3.1 [95 % CI, 1–9.3], respectively). Considering the DFS multivariate analysis, only the presence of skin ulceration (HR=7.8 [95 % CI 1.2-

Table 4 Location and number of metastases found at necropsy of 23 cats with mammary carcinoma

Metastases location	Number of animals (%)
Lung and/or pleura	20 (87.0 %)
Non-regional lymph nodes	16 (70.0 %)
Liver	7 (30.4 %)
Skin	5 (21.7 %)
Urinary system	4 (17.4 %)
Cardiac muscle and/or pericardium	3 (13.0 %)
Muscle	3 (13.0 %)
Pancreas	3 (13.0 %)
Spleen	2 (8.7 %)
Adrenal glands	2 (8.7 %)
Thyroid gland	2 (8.7 %)
Gynae organs	2 (8.7 %)
CNS	1 (4.3 %)
Number of metastases per animal	Number of animals (%)
0–2	8 (34.8 %)
3–5	10 (43.5 %)
6–8	5 (21.7 %)

Table 5 Multivariate cox regression analysis for OS in cats that underwent surgery only (n=42)

	Overall survival				
	n	HR[5]	95 % CI[6]		p
			Lower	Upper	
Breed	42	0.13	–	–	0.135
Disease stage (TMN)	42	4.1	1	17.1	0.05
Tumor size					
<2 cm	10	0.023	0.002	0.2	0.001*
2–3 cm	19	3.1	1	9.3	0.048*
>3 cm	13	*Reference*			
MG[1] (Mills et al.[27] system)	42	0.2	0.01	1.9	0.148
Lymphatic invasion	42	3.9	0.7	21.2	0.114
ER status	42	2.1	0.3	15.9	0.482
CK5/6 status	42	0.8	0.2	3.5	0.76
Ki-67 index					
Low	14	*Reference*			
High	28	34.6	1.3	926.5	0.035*
Molecular Classification					0.009*
Luminal A	8	3.5	0.2	106.4	0.467
Luminal B/HER2-[2]	14	55.9	3.5	886.5	0.004*
Luminal B/HER2+[3]	9	55.7	2.1	1467.8	0.016*
HER2-positive	3	438.4	14	13681.7	0.001*
TN[4] basal-like	4	210.9	4.8	9352.3	0.006*
TN[4] normal-like	4	*Reference*			

MG malignancy grade, *Luminal/HER2−* Luminal B/HER2 negative, *Luminal/HER2+* Luminal B/HER2 positive, *TN* triple negative, *HR* hazard ratio, *CI* confidence interval

Table 6 Multivariate cox regression analysis for DFS in cats that underwent surgery only (n=36)

	Disease-free survival				
	n	HR[5]	95 % CI[6]		p
			Lower	Upper	
Disease stage (TMN)	36	1.2	0.5	3.1	0.686
Tumor size	36	1.3	0.5	3.3	0.608
MG[1] (Mills et al. [27] system)	36	1.5	0.4	5.6	0.581
Necrosis	36	3.2	0.7	14.2	0.118
Lymphocytic invasion	36	4.9	0.9	27.6	0.078
Ulceration					
No	30	*Reference*			
Yes	6	7.8	1.2	52.4	0.034*
Molecular Classification					0.398
Luminal A	8	.32	0.009	10.9	0.528
Luminal B/HER2-[2]	13	1.4	0.1	18.4	0.787
Luminal B/HER2+[3]	8	1.1	0.1	13.4	0.918
HER2-positive	2	2.1	0.2	22.6	0.531
TN[4] basal-like	3	10.5	0.3	326.2	0.181
TN[4] normal-like	2	*Reference*			

MG malignancy grade, *Luminal/HER2−* Luminal B/HER2 negative, *Luminal/HER2+* Luminal B/HER2 positive, *TN* triple negative, *HR* hazard ratio, *CI* confidence interval

52.4], p=0.034) has proven to be an independent predictor. No other variables independently affected OS or DFS in the multivariate survival study.

Associations and concordance of the expression pattern of biomarkers

Estrogen receptor (ER)

In primary tumors (PT), ER positivity was found to be associated with early stages of the disease (TNM I and II, p=0.04) and with local relapse (p=0.025), whereas ER-negative mammary carcinomas were associated with lymphatic vessel invasion by neoplastic cells (p= 0.011), Ki-67 index≥14 % (p= 0.035), larger tumors (p= 0.048), high-grade carcinomas (p= 0.003), and with the presence of metastases in regional lymph nodes (p=0.038, supplementary Table 2). ER-negative relapse lesions were significantly associated with grade III tumors (p=0.02), with the presence of necrosis (p=0.007) and lymphatic invasion (p=0.041, supplementary Tables 3 to 5).

The overall concordance of ER status between the PT and LR was moderate (56.3 %, 9/16), although smaller than between PT and RM (91.2 %, 31/34) and between PT and DM (84.6 %, 11/13). 40 % (2/5) of the animals with ER-positive PT showed low expression levels of ER in metastases, in contrast with the low percentage of cats (6.5 %, 2/31) which showed ER-negative mammary carcinomas coupled with one or more ER-positive metastases.

Progesterone receptor (PR)

The analysis of the expression pattern of PR revealed that 18.2 % (4/22) of the animals with PR-positive mammary carcinomas showed PR-negative metastatic lesions, which was very similar to the 28.6 % (4/14) of cats with PR-negative PT and PR-positive metastases. Further immunohistochemical analysis demonstrated that the majority of PR-positive primary and metastatic lesions were ER-negative. Indeed, 84.4 % (27/32) of cats with PR-positive and ER-negative PT, 72.7 % (8/11) showed PR-positive and ER-negative LR, 91.7 % (22/24) had PR-positive and ER-negative RM, and 100 % (7/7) showed PR-positive and ER-negative DM.

Mammary carcinomas with PR overexpression were significantly associated with neutered cats (p=0.049), while PR-negative tumors were associated with CK5/6 negative status

(p=0.034). Further, PR-positive recurrent lesions were significantly associated with the presence of necrotic areas (p= 0.035, supplementary Tables 2 to 5).

The concordance of PR status in PT and LR was 68.8 % (11/16), 82.4 % (28/34) between PT and RM, and 61.5 % (8/13) between PT and DM.

fHER2

Immunohistochemical analysis revealed that 31.1 % (19/61) of the cats had *f*HER2-positive carcinomas, 42.9 % (9/21) showed *f*HER2-positive relapses, 25 % (12/48) exhibited *f*HER2-positive regional metastases, and 26.1 % (6/23) had *f*HER2-positive distant metastases (Table 3).

Slightly more than half of the animals with *f*HER2-positive mammary tumors had *f*HER2-negative metastatic lesions (54.5 %, 6/11), contrasting with the small percentage of animals that showed *f*HER2-negative mammary carcinoma and *f*HER2-positive metastatic lesions (16 %, 4/25). A concordance of 68.8 % (11/16) was found for the *f*HER2 status between PT and LR, similarly to the concordance ratio between PT and RM (67.6 %, 23/34), but lower than the concordance rate found between PT and DM (84.6 %, 11/13). *f*HER2 did not present significant associations with the other clinicopathological features (supplementary Tables 2 to 5).

Cytokeratin 5/6 (CK5/6)

The percentage of CK5/6-positive samples was identical for primary tumors and metastases (Table 3). Indeed, 48.3 % (29/61) of the cats with mammary carcinomas had CK 5/6-positive tumors, 47.6 % (10/21) had CK 5/6-positive local relapses, 54.2 % (26/48) showed CK 5/6-positive regional metastases and 43.5 % (10/23) had CK 5/6-positive distant metastases.

Interestingly, when the animals that underwent surgery without chemotherapy were analyzed, the percentage of cats with CK5/6-negative mammary carcinoma that showed CK5/6-positive metastatic lesions was identical (33.3 %, 6/18) to the percentage of cats that showed CK5/6-positive tumors and CK5/6-negative metastatic lesions (33.3 %, 6/18).

Only PT showed significant associations between CK5/6 expression and clinicopathological features (supplementary Tables 6 to 9), with CK5/6-positive PT being associated with the presence of multiple mammary carcinomas (p= 0.032), grade III tumors (Elston & Ellis classification, p= 0.044) and PR-positive status (p= 0.034), whereas CK5/6-negative carcinomas were associated with the absence of lymphatic vessel invasion (p= 0.05). The concordance of results for CK5/6 status was of 56.3 % between mammary carcinomas and LR (9/16), slightly lower than between the PT and RM (73.5 %, 23/34), but identical to the concordance between PT and DM (53.8 %, 7/13).

Ki-67 index

Analysis of Ki-67 expression levels showed a trend towards a higher mean throughout the disease progression, with primary tumors and local relapses displaying lower mean Ki-67 index (26.7 and 22.9 %, respectively) than regional (32.2 %) and distant metastases (36 %). In fact, 77 % (47/61) of PT and 71.4 % (15/21) of RL showed a Ki-67 index≥14 %, whereas 83.3 % (40/48) of RM and 91.3 % (21/23) of DM also showed a Ki-67 index value equal or higher than 14 %. We also observed a moderate concordance between the Ki-67 index of PT and LR (43.8 %, 7/16) and high concordance rates between PT and RM (73.5 %, 25/34), and between PT and DM (76.9 %, 10/13). When a threshold value of 14 % was used, 80 % (8/10) of cats that showed Ki-67-negative mammary carcinoma had Ki-67-positive relapse/metastatic lesions, in contrast to only 15.4 % (4/26) of animals that showed Ki-67 positive mammary tumor and Ki-67-negative relapse/metastatic lesions. Finally, mammary carcinomas with a Ki-67 index≥14 % showed a significant association with large tumor size (p= 0.001), high-grade carcinomas (according to Mills et al. classification, p<0.0001), presence of necrotic areas (p= 0.011) and with ER-negative expression (p= 0.035, supplementary Tables 6 to 9).

Molecular subtypes

The molecular immunophenotyping of all mammary carcinomas (n=61), local relapses (n=21), regional (n=48), and distant metastases (n=23) was performed using the St. Gallen's guidelines. Our results demonstrate that protein expression patterns could be different between PT, LR, RM, and DM, for the same animal. Indeed, luminal B (HER2− and HER2+) was the most common subtype in PT (52.5 %; 32/61), while 65.2 % (15/23) of DM were triple negative (basal and normal-like). Moreover, all cats with luminal A primary carcinomas (n=8) showed more aggressive subtypes in all metastatic lesions. Remarkably, the luminal B/HER2-positive subtype was well conserved between primary tumors and paired metastases (75 %; 6/8), followed by the luminal B/HER2-negative subtype (63.6 %; 7/11), by triple negative/basal-like subtype (50 %; 2/4), by HER2-positive subtype (33.3 %; 1/3) and by the triple negative normal-like subtype (25 %; 1/4). Additional statistical analysis revealed that only LR showed significant associations with lymphatic vessel invasion by neoplastic cells (p= 0.032, supplementary Tables 10 and 11). In fact, we found that triple negative

subtypes were significantly associated with the presence of lymphatic invasion ($p = 0.032$), while both luminal A and luminal B (HER2-negative and HER2-positive) tumors were associated with the absence of lymphatic vessel invasion in local relapses.

In summary, the maintenance of the tumor subtype through disease progression was relatively low, with a concordance rate of 31.3 % (5/16) between the PT and the LR, of 47.1 % (16/34) between the PT and the RM, and of 53.8 % (7/11) between the PT and the DM.

Discussion

The post-mortem examination of 23 queens diagnosed with MC revealed that the lungs and/or pleura were the most common organs involved in metastatic disease (87 %), followed by the non-regional lymph nodes (70 %) and the liver (30.4 %), which is similar to what is reported in humans [35]. At the moment of death, most of the animals had more than one organ invaded (87 %, 20/23), thus revealing the high metastatic potential of this carcinoma type.

Our results also demonstrated that the majority of metastases showed loss of ER and PR expression compared to the corresponding primary tumors, as equally reported in patients with breast cancer [9, 36, 37]. This phenomenon was more evident for ER expression, with cats having ER-positive mammary tumors being associated with a better outcome, also as reported for women with breast cancer [38–40]. In addition, PR-positive mammary carcinomas were associated with neutered cats at the time of mastectomy. This can be explained by the fact that most of the animals were already spayed during adulthood, and were therefore exposed to the ovarian hormones most of their life, explaining the association found between these two parameters [1, 14]. Moreover, PR-positive LR were correlated with the presence of necrotic areas. Our data are consistent with findings reported for breast cancer patients, where, primary ER−/PR+ breast cancers exhibit a more aggressive behavior than ER+ or ER+/PR+ tumors [41, 42].

Low concordance rates in *f*HER2 status were found between primary and relapse lesions (68.8 %) and between primary tumors and regional metastases (67.6 %), similarly to what is found in breast cancer patients [6, 43]. This study also revealed that cats with CK5/6-positive mammary carcinomas were associated with poor prognostic features and lower overall survival time, as described for breast cancer patients [44–46]. Additionally, CK 5/6-positive mammary carcinomas were found to be correlated with the presence of multiple and poorly differentiated primary tumors, with PR-positive status, whereas CK 5/6-negative FMCs were associated with the absence of lymphatic vessel invasion.

Evaluation of Ki-67 expression showed that metastases had higher indexes than paired mammary carcinomas. Ki-67 expression was also associated with worse outcome features (larger tumor, high-grade carcinomas, presence of necrosis, and ER-negative expression), corroborating our data which significantly correlate lower OS rates with cats having high Ki-67 index mammary tumors, either in univariate or in multivariate survival analysis, which is also reported for breast cancer patients [35, 47–49].

Finally, the luminal B/HER2-negative and the luminal B/HER2-positive were the most frequent subtypes out of the six evaluated, corroborating the data published by Brunetti et al. [22]. In parallel, the prevalence of the triple negative/basal-like FMC (16.4 %) was lower than reported by Maniscalco et al. [50] and Wiese et al. [51], even though the frequency of the triple negative/basal-like metastases was higher (39.1 %). These results are relevant as cats with basal-like mammary carcinomas are associated with lower OS and DFS periods, and larger tumors with lymphatic vessel invasion, as women with triple-negative/basal-like breast cancer [45]. Moreover, molecular classification has proved to be an independent prognostic factor related to OS, with an animal with HER2-positive MC displaying a 438-fold increased risk of tumor-related death, in comparison with the other subtypes and the triple negative/basal-like mammary carcinomas presenting a hazard ratio of 210.8.

Altogether, our results disclose the high metastatic capacity of FMC and the heterogeneity of protein expression profiles between primary tumors and paired metastases. However, the majority of local relapses had less aggressive features when compared to paired primary tumor (smaller size, grade I tumors, absence of necrosis, and vascular invasion). From a molecular point of view, relapse lesions also showed a higher percentage of ER-positive cases, a lowest Ki-67 mean index and a low percentage of triple negative/basal-like cases. We hypothesize that these findings could have resulted from a greater awareness and a closer follow-up of the animals before mastectomy, which enabled the early detection of disease relapses and, consequently, resulting in less aggressive characteristics.

Conclusions

Taking our results in consideration on the prognostic value of each molecular subtype, the FMC immunophenotyping should be routinely performed in order to choose the better therapeutic strategies, once FMC subtypes mimic breast cancer subtypes. The presence of metastases must also be carefully examined to improve the clinical management of the animal, considering that DM usually show a worse immunophenotype than

matched primary tumor/local metastases probably due to an adaptive response to different microenvironments found in diverse tissues. Finally, the similarities found between FMC and human breast cancer highlight the fact that FMC can be used as a model for comparative oncology.

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Compliance with ethical standards Tumor samples were collected in accordance with the EU Directive 2010/63/EU, and research was approved by the ethics committee of the Faculty of Veterinary Medicine (FVM), University of Lisbon (ULisboa).

Conflicts of interest None

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ANNEX V – Serum HER2 levels are increased in cats with mammary carcinomas and predict tissue HER2 status

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Serum HER2 levels are increased in cats with mammary carcinomas and predict tissue HER2 status

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ABSTRACT

HER2 is overexpressed in about 30% of feline mammary carcinomas (FMC) and in 15-30% of breast cancers. Women with HER2-positive breast tumors are associated with shorter survival. This study aimed to optimize the detection and quantification of serum HER2 (sHER2) in cats and to evaluate its potential in diagnosing cats with mammary carcinomas (MC) overexpressing HER2. A prospective study was conducted in 60 queens showing MC and 20 healthy animals. Pre-operative serum samples were collected for sHER2 quantification using two immunoassays: ELISA and Dot blot assay. sHER2 levels were compared with tissue HER2 status assessed by immunohistochemistry. Queens with FMC showed significantly higher mean levels of sHER2 by both ELISA and Dot blot assay. A significant difference in the sHER2 levels was also found between cats with HER2-positive MC and those with low-expressing HER2 MC. A significant correlation between sHER2 levels and tumor HER2 status was also found, particularly when ELISA was used ($r = 0.58$, $p < 0.0001$). The value of 10 ng/ml was proposed as the optimal cutoff for both immunoassays by ROC analysis. Like in humans, sHER2 levels are increased in cats with MC HER2-positive, strongly suggesting that evaluation of sHER2 levels can be very useful in feline oncology. The results show that ELISA and Dot blot assay can replace the immunohistochemistry technique, due to their efficacy and lower costs for diagnostic purposes and for monitoring the response to anti-HER2 therapies in cats.

INTRODUCTION

Animal models have been crucial in increasing our understanding of tumorigenesis, with mouse species being the most commonly used because of their small size and short gestation period [1]. However, due to several limitations in using laboratory rodents, prevailing tumors in pets appear to be good alternative models, especially because of their high incidence. Moreover, pets also share a similar environment to humans and show a more similar body size, thus facilitating the pharmacokinetic and toxicological studies and shortening the phase I trials in humans [1-5].

Feline mammary carcinomas (FMC) are very common in cats, showing an incidence that ranges from 12 to 40% of all tumors [6, 7]. Their epidemiological and histopathological features closely resemble those found in the more aggressive breast cancer types [8]. Thus, it has been proposed as a putative model for cancer studies [2, 8-13]. Recently, some reports have shown that the feline homologue of human epidermal growth factor receptor-2 proto-oncogene (HER2) is overexpressed in 33%-60% of FMC [10, 13, 14, 15] and it was associated with a shorter overall survival (OS) [14] as in human HER2 positive breast tumors, even though the HER2 gene amplification could not be detected [10, 13].

HER2 has a molecular mass of 185 kDa and is a transmembrane glycoprotein which comprises three domains: an extracellular domain (ECD), a short transmembrane region and an intracellular domain with tyrosine kinase activity [16-18]. In women, the gold standard method to identify HER2-positive breast tumors is immunohistochemistry (IHC), with fluorescence in situ hybridization (FISH) used additionally to identify HER2

gene amplification status in ambiguous cases [19, 20]. Nevertheless, several limitations, like the impossibility of conducting continuous follow-up after the invasive surgery and the high costs of the reagents, has led to the development of new non-invasive techniques to quantify HER2 in serum (sHER2). The HER2-ECD is shed from the surface of tumor cells into the bloodstream via protease activity (A Disintegrin And Metalloproteinase domain-containing proteins - ADAMs family), allowing its detection in sera by ELISA or by a chemiluminescence method [21]. In fact, many studies have found that breast cancer patients with elevated HER2-ECD levels were associated with higher relapse rates and worse prognosis [21-23]. More recently, it was reported that the Dot blot assay could also be used to measure sHER2 levels, being a less expensive method and representing a good alternative to closely follow the tumor disease progression [21, 24, 25].

To the best of our knowledge, no studies have been published on the utility of assessing sHER2 levels in small animals. Thus, the main objectives of this study were: i) to accurately quantify the sHER2 levels in cats using both ELISA and the Dot blot assay; ii) to evaluate the usefulness of measuring sHER2 levels in the diagnosis of FMC overexpressing HER2; and iii) to determine the optimal cutoff value for the ELISA and the Dot blot assay, that would differentiate cats with mammary carcinoma overexpressing HER2 from cats with HER2-negative mammary carcinomas or healthy animals.

RESULTS

Animal study population

A total of sixty queens displaying mammary carcinoma were used in the study. Their main clinicopathological characteristics are summarized in Table 1. The mean age at diagnosis was 11.57 years (range, 7-17 years) and all animals were treated surgically, except one, which displayed pulmonary metastasis. Fifty (83.3%) cats were subjected to unilateral mastectomy, six (10%) to bilateral mastectomy and the remaining 3 (6.7%) to regional mastectomy. After surgery, five of these queens were subjected to anthracycline-based adjuvant chemotherapy (doxorubicin, 25 mg/m^2, intravenously, every 3 weeks for 5 cycles). Tumor samples from the untreated animal were collected after euthanasia.

The mean size of the primary mammary carcinomas was 2.33 cm (range 0.3-7 cm) and the mean value of Ki-67 proliferation index was 19.5% (ranging between 1.2% and 46%). Twenty two (36.7%) cats showed HER2-overexpressing MC (assessed by IHC), out of which nine (15%) were classified with a 3+ and thirteen (21.7%) with a 2+ score (Table 1).

Survival data for disease free-survival (DFS) were available for 52 queens. Sixty-four percent (33/52) of the cats with mammary carcinoma showed disease recurrence at the end of the follow-up period (2 years and 2 months), with the majority showing locoregional relapses (24/33, 72.7%) and the rest distant metastases (27.3%, 9/33). The mean DFS was 13.4 ±1.82 months (95% CI: 9.8-17 months). Regarding the overall survival (OS), data were available for 58 animals, from which twenty-nine (50%) died, presenting a mean survival of 27.18 ±3.4 months (95% CI: 20.5-33.9 months).

Cats with mammary carcinoma show elevated sHER2 levels

Serum HER2 levels were measured by both ELISA and Dot blot assay. The circulating HER2 levels were calculated from the standard curve of known rHER2-ECD concentrations (Figure 1). The intra-assay and inter-assay

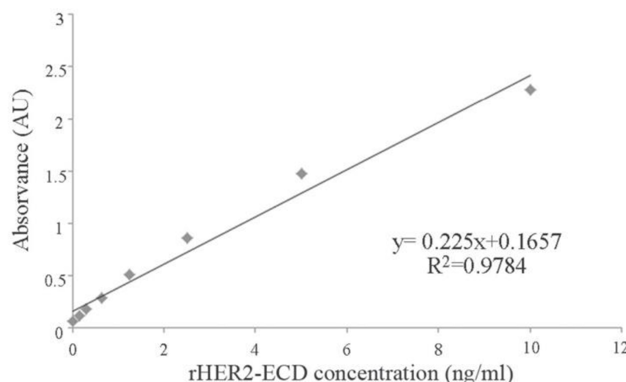

Figure 1: Standard curve for sHER2 measurements using a commercial ELISA kit. The intra and inter-assay coefficients of variation were < 10%.

Table 1: Clinicopathological features of 60 female cats with mammary carcinoma

Clinicopathological feature	Number of animals (%)	Clinicopathological feature	Number of animals (%)
Breed		**Size**	
Not determined	46 (76.7%)	<2 cm	24 (40.0%)
Siamese	9 (15.0%)	2-3 cm	24 (40.0%)
Persian	3 (5.0%)	>3 cm	12 (20.0%)
Norwegian Forest Cat	**2 (3.3%)**	**[a]HP classification**	
Spayed		Tubulopapillary carcinoma	36 (60.0%)
No	30 (50.0%)	Solid carcinoma	13 (21.7%)
Yes	29 (48.3%)	Cribriform carcinoma	6 (10.0%)
Unknown	1 (1.7%)	Mucinous carcinoma	5 (8.3%)
Contraceptives		**Malignancy grade**	
No	18 (30.0%)	I	3 (5.0%)
Yes	31 (51.7%)	II	14 (23.3%)
Unknown	11 (18.3%)	III	43 (71.7%)
Treatment		**Necrosis**	
None	1 (1.7%)	No	20 (33.3%)
Mastectomy	54 (90.0%)	Yes	40 (67.7%)
Mastectomy + Chemo	5 (8.3%)	**Lymphatic invasion**	
Multiple tumors		No	49 (81.7%)
No	22 (36.7%)	Yes	11 (18.3%)
Yes	38 (63.3%)	**Lymphocytic infiltration**	
Lymph node status		No	24 (40.0%)
Negative	32 (53.3%)	Yes	36 (60.0%)
Positive	23 (38.3%)	**Tumor ulceration**	
Unknown	5 (8.3%)	No	57 (95.0%)
Stage (TNM)		Yes	3 (5.0%)
I	13 (21.7%)	**Ki 67 index**	
II	9 (15.0%)	Low (< 14%)	24 (40.0%)
III	29 (48.3%)	High (≥ 14%)	35 (58.3%)
IV	9 (15.0%)	Unknown	1 (1.7%)
Localization		**PR status**	
M1	9 (15.0%)	Negative	28 (46.7%)
M2	12 (20.0%)	Positive	32 (53.3%)
M3	24 (40.0%)	**ER status**	
M4	14 (23.3%)	Negative	40 (33.3%)
Unknown	1 (1.7%)	Positive	20 (66.7%)
		***f*HER2 status**	
		Negative	38 (63.3%)
		Positive	22 (36.7%)

[a]HP classification, Histopathological classification

Table 2: Serum HER2 levels in cats with mammary carcinoma

Immunoassay	Cats (n, %)	Mean and range values (ng/ml)
ELISA (*n* = 40)		
Low levels (< 10 ng/ml)	24 (60.0%)	0.35 (0.00-8.38)
High levels (≥ 10 ng/ml)	16 (40.0%)	55.17 (13.75-147.05)
Dot blot assay (*n* = 47)		
Low levels (< 10 ng/ml)	20 (42.6%)	2 (0-5)
High levels (≥ 10 ng/ml)	27 (57.4%)	22.78 (10-75)

coefficients of variation were 6.2% and 4.6%, respectively.

Box plots were used to illustrate the full data range of each group and to identify outliers. Cats having sHER2 levels that fall more than three standard deviations away from the mean were considered outliers and removed from further analysis. ELISA results gave two outliers in the healthy group and seven in the cancer group that were removed. There were no outliers in the Dot blot results.

ELISA results revealed that cats from the cancer group showed significantly higher sHER2 levels (mean = 22.3 ng/ml; range of values: 0-147.05 ng/ml) than healthy animals (mean = 18.3 ng/ml; range of values: 0-104.2 ng/ml), with a significant *p*-value of 0.04 (Figure 2A). A more significant difference between the sHER2 levels of the two animal groups was found when Dot blot assay was performed ($p = 0.01$), with the cancer group showing a mean value of sHER2 of 14 ng/ml (range of values: 0-75 ng/ml) and the healthy group a mean value of 4.5 ng/ml (range of values 0-15 ng/ml), as depicted in Figure 2B.

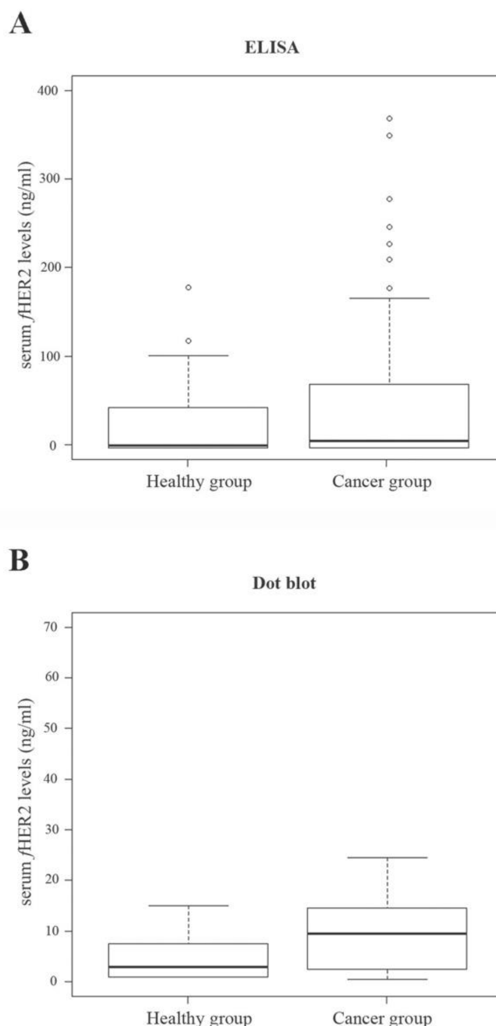

Figure 2: Box plot diagrams representing the sHER2 levels in control cats (healthy group) and in cats with mammary carcinoma (cancer group) determined by ELISA (A) and Dot blot assay (B). Cats with mammary carcinomas had significantly higher mean of sHER2 levels than healthy cats both by ELISA ($p = 0.04$) and Dot blot assay ($p = 0.01$). Outliers are indicated by open circles.

Serum HER2 levels predict the tumor HER2 status

A significant difference was found between sHER2 levels of cats with HER2-negative mammary carcinoma (IHC-negative group) and sHER2 levels of cats diagnosed with mammary carcinoma overexpressing HER2 (IHC-positive group), by both ELISA ($p = 0.001$, Figure 3A) and Dot blot assay ($p = 0.03$, Figure 3B). Additionally, a strong correlation was found between sHER2 levels quantified by ELISA and tumor HER2 status (r = 0.58, $p < 0.0001$), coupled with a moderate association between sHER2 levels measured by Dot blot assay and tumor HER2 status (r = 0.26, $p < 0.1$). Moreover, the Kappa coefficient showed a moderate agreement between ELISA and IHC results (k = 0.48, p = 0.002), and a good agreement between Dot blot assay and IHC results (k = 0.264, p = 0.047). However, results obtained by ELISA and Dot blot assay only showed a fair agreement (k = 0.27, p = 0.048).

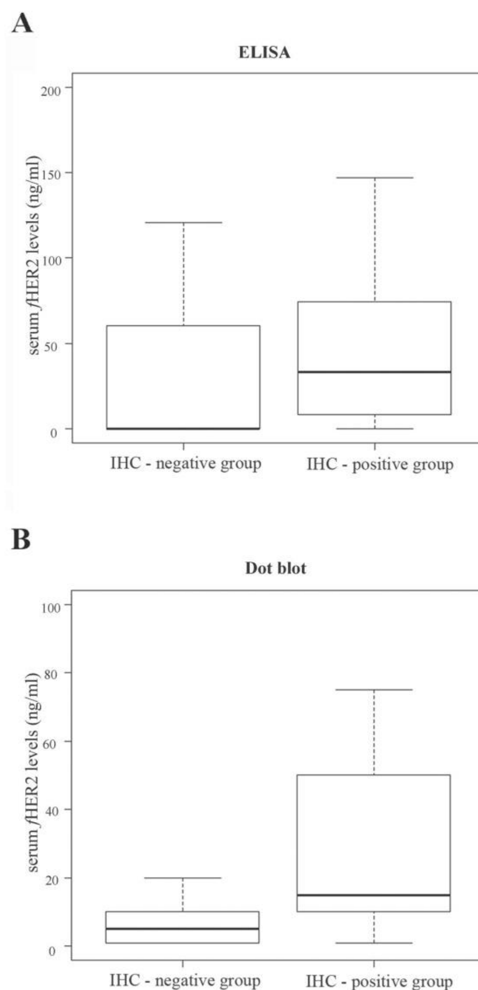

Figure 3: Box plot diagrams showing that tumor HER2 status correlates with sHER2 levels as assessed by both ELISA (A) and Dot blot assay (B). A. A significant difference was found ($p = 0.001$) between the sHER2 levels of cats with MC overexpressing HER2 (IHC-positive group: mean 42.6 ng/ml [range of values 0-147.05 ng/ml]) and the sHER2 levels of cats with HER2-negative MC (IHC-negative group: mean 8.8 ng/ml [range of values 0-73.89 ng/ml]), using ELISA ($p = 0.001$). **B.** The Dot blot assay also showed a significant difference between the sHER2 levels measured in these two studied groups, with a p-value of 0.03 (IHC-positive group: mean 24.4 ng/ml [range of values 0-75 ng/ml]; IHC-negative group: mean 8 ng/ml [range of values 0-20 ng/ml]).

Serum HER2 molecules contain a portion of the intracellular receptor domain

In order to validate the measurement of sHER2 levels by the Dot blot assay, the antigenic specificity of the anti-HER2 monoclonal antibody (clone SP3) was evaluated through western blot analysis. As expected, the SP3 antibody recognizes a protein band of ~185 kDa corresponding to predicted molecular weight of full-length HER2 in whole cell extracts of both human breast cancer cells (SKBR3, Figure 4A, line 1) and feline mammary tumor cells (FMCp, Figure 4A, lane 2), and cross-reacts with the recombinant human HER2-ECD (Figure 4A, lane 3, 84-90 kDa). Further western blot analysis revealed that the anti-HER2 monoclonal antibody detected several protein bands with masses ranging from 120 to 140 kDa in the serum samples from cats diagnosed with MC overexpressing HER2 (Figure 4A, lanes 4 and 5), suggesting that proteolytic cleavage of HER2 occurs at the intracellular domain (ICD), leading to the production of truncated HER2 soluble forms which are quantifiable by the Dot blot assay (Figure 4B). As mentioned above, cats diagnosed with HER2-overexpressing MC (scored as 2+ and 3+), showed significantly ($p = 0.03$) higher sHER2 levels (Figure 4B, lines 5 and 6) than cats with HER2-negative mammary carcinomas (Figure 4B, lines 3 and 4) or healthy cats (Figure 4B, line 2).

sHER2 levels ≥10 ng/ml give the optimal cutoff value to diagnose HER2-overexpressing FMC

To determine the best cutoff value, the sensitivity and the specificity of both ELISA and Dot blot assay were determined using different cutoff points to classify cats according to their sHER2 levels. ROC curve analysis revealed that the best cutoff was ≥10 ng/ml to discriminate cats with mammary carcinomas overexpressing HER2 (Table 2). By using this threshold value, the sensitivity and the specificity of ELISA were 69% and 67%, respectively (Figure 5A, [AUC = 0.70, 95% CI = 0.55-0.85]), while the Dot blot assay revealed a sensitivity of *53%* and a *specificity of 78%* (Figure 5B, [AUC, 0.73; 95% CI = 0.58-0.88]).

Six out of twenty healthy cats (30%) had elevated sHER2 levels (≥10 ng/ml) when ELISA was used and 5/20 (25%) with the Dot blot assay. This is in accordance with studies in humans, where false-positive rates can reach up to 20% [25].

Elevated sHER2 levels are associated with a less aggressive tumor phenotype

ELISA results revealed that cats with sHER2 levels ≥10 ng/ml were significantly associated with the early stage of the disease (stage I, $p = 0.006$; Odds Ratio [OR] = 17.9; 95% CI: 1.633-984.42), absence of tumor necrosis ($p = 0.0024$; OR = 9.28; 95% CI: 1.79-60.61) and lower Ki-67 values (< 14%, $p = 0.007$; OR = 7.73; 95% CI: 1.57-47.54). In parallel, the Dot blot assay results also

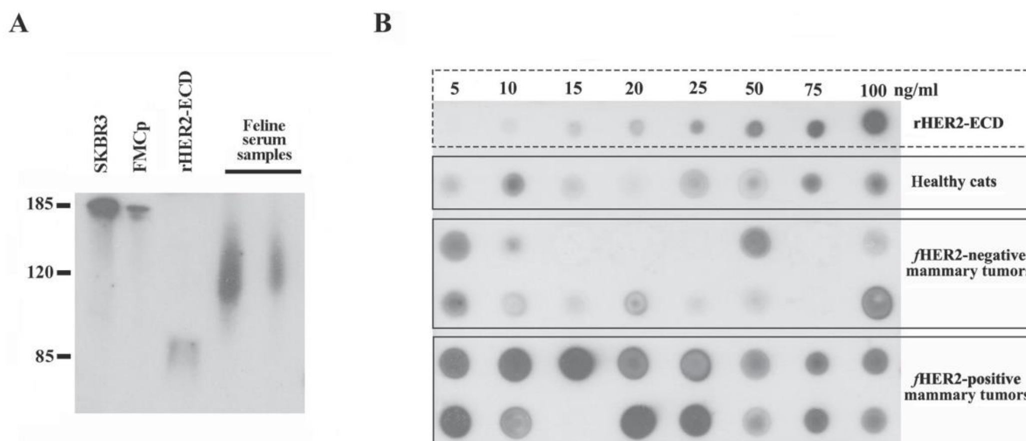

Figure 4: Soluble truncated HER2 forms carry a portion of the ICD and are quantifiable by Dot blot assay. A. The anti-HER2 SP3 antibody specifically recognizes a protein band of about 185 kDa in human and feline whole cell extracts (SKBR3 and FMCp, lanes 1 and 2), the purified rHER2-ECD (lane 3, ~75 kDa), and a protein with a molecular weight that ranges from 100 to 140 kDa in cat serum samples (lanes 4 and 5). **B.** A representative image of an immunoblot performed with serum samples collected from healthy cats (line 2) and cats with mammary carcinoma (lines 3-6). sHER2 levels were semi-quantified by comparing the intensity of each serum dot with the intensities of the dots obtained from rHER2-ECD dilutions (line 1).

correlate with high sHER2 levels (≥10 ng/ml) with cats that have mammary carcinomas with low Ki-67 index (p = 0.016; OR = 5.24; 95% CI: 1.23-27.79). Supporting the above results, significant correlations were found between the HER2-negative tissue status and the presence of tumor necrosis (p = 0.0012; OR = 7.16; 95% CI: 1.91-30.5), moderate malignancy grade (p = 0.049; OR = 3.4; 95% CI: 0.8-14.6), low Ki-67 values (p = 0.0022; OR = 6.42; 95% CI: 1.81-25.5) and ER positivity (p = 0.049; OR = 3.15; 95% CI: 0.91-11.45).

DISCUSSION

In this study, 36.7% (22/60) of the selected population had HER2-overexpressing mammary carcinomas, corroborating previously published data [8, 10, 13, 14].

To the best of our knowledge, this is the first work to detect and quantify the serum HER2 levels in cats, using two immunoassays: the ELISA which has already been approved to measure sHER2 levels in women with metastatic breast cancer, and the Dot blot assay, a less expensive technique [24, 26]. Our results show

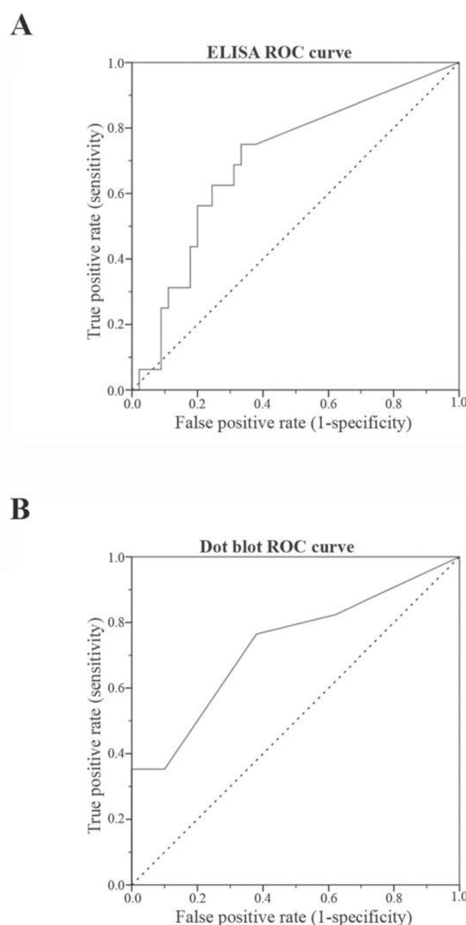

Figure 5: Receiver-operating characteristic (ROC) curve for sHER2 levels for ELISA (A) and Dot blot assay (B). The optimal sHER2 cutoff value (10 ng/ml) was chosen to maximize the sum of the sensitivity and specificity on the Youden index (sensivity+specifity-1). The AUC was 0.70 (95% CI: 0.55-0.85) for ELISA **A.** and 0.73 (95% CI: 0.58-0.88) for Dot blot assay **B.**, demonstrating that the sHER2 levels are a good biomarker to differentiate cats with MC overexpressing HER2 from cats with MC non-expressing HER2 or healthy cats. When the cutoff value of 10 ng/ml was set, the sensibility was 69% and the specificity was 67% for ELISA, whilst for Dot blot assay the sensibility and the specificity were 53% and 78%, respectively.

that sHER2 levels in cats with HER2 overexpressing mammary carcinomas are significantly higher than in cats with HER2-negative mammary carcinomas ($p = 0.001$) or healthy animals ($p = 0.04$). As also reported for breast cancer patients, a false-positive rate of 25-30% was found in healthy cats, when either ELISA or Dot blot assay was performed [21, 25, 27].

Using ROC analysis the best cutoff value to identify cats with mammary carcinomas overexpressing *f*HER2 was 10 ng/ml in both techniques. Interestingly, this threshold is also used in woman with breast cancer [25, 28]. Comparing the two assays, *ELISA was* more *sensitive* in detecting cats with elevated sHER2 levels than Dot blot assay (69% versus 53%), but less specific (67% *versus* 78%).

Moreover, a moderate correlation was found between the sHER2 levels and the tissue HER2 status when ELISA was employed ($r = 0.58$, $p < 0.0001$), revealing that sHER2 levels predict the HER2 status of the primary mammary tumor. However, the correlation with the Dot blot assay was low ($r = 0.3$). ELISA studies in humans showed contradictory results: some did not find any correlation between tissue and serum HER2 levels [29], whereas others reported a poor or a moderate/strong correlation [21, 23, 25, 30]. Regarding the Dot blot assay technique, there is only one study that correlates the tissue and serum HER2 levels, giving a similar result to this study ($r = 0.3$) [24].

Western blot analysis demonstrated that the soluble HER2 fragments also contain a portion of the intracellular domain, since a protein band between 100 and 135 kDa was detected. This result was additionally supported by the use of two anti-HER2 antibodies that recognize the intracellular domain of HER2 (4B5 and A0485) in the Dot blot assay, where a similar signal intensity to the SP3 antibody was obtained.

Further statistical analysis revealed that cats with MC showing HER2 overexpression or high sHER2 levels were associated with features usually associated with a better outcome (low Ki-67 index, absence of tumoral necrosis, ER-positive status and lower disease stages). Previous studies in cats did not show significant associations [9, 14, 31]. Although HER2-positive tumors are associated with aggressive tumoral features [21, 23, 32], our results could be due to the fact that most of HER2-overexpressing FMC also presented a positive ER and/or PR status, being classified as luminal B and not as HER2-positive immunophenotype. Indeed, luminal B breast tumors in humans showed a less aggressive behavior than HER2-positive tumors [33].

In summary, this study revealed that cats with mammary HER2-overexpressing carcinomas showed elevated sHER2 levels, which can be detected and quantified by ELISA and Dot blot assay. Even though more studies are needed to clarify the role of HER2 in the pathogenesis of FMC, our results showed that sHER2 level measurements are useful to diagnose FMC overexpressing HER2, and probably, to predict the therapeutic response to anti-HER2 agents (antibodies or kinase inhibitors), as in humans, where a rapid decrease of the sHER2-ECD levels after treatment is indicative of a good therapeutic response [21, 34, 35].

This study has also shown that *f*HER2 protein can release its extracellular domain into the extracellular space, an event that is apparently similar to the one described in humans. ELISA appears to be the best technique, giving a higher correlation with the tissue level and although less specific than Dot blot assay, could be a supplement to the IHC with possible applications in Veterinary Medicine. High serum *f*HER2-ECD levels are present in 40% of the feline patients (using the ELISA assay) and overexpression of *f*HER2 protein was observed in 36.7% of the population (with IHC).

Surprisingly, the *f*HER2 protein in serum and in tissue was associated with less aggressive features, contradicting what is described for humans. These findings, together with the non-amplification of the *f*HER2 gene [13], reinforce the need for more studies in order to clarify the biological role of this protein.

Finally, the high prevalence of this protein in FMC could be important for the study of specific target therapies directed against HER2 protein, both in a veterinary clinical perspective, improving the therapeutic options of these animals and also in a comparative perspective, using the cat as a model for therapeutic trials.

MATERIALS AND METHODS

Cat study population

Sixty female cats with spontaneous mammary carcinomas that underwent surgical treatment at the Small Animal Hospital of the Veterinary Medicine Faculty, University of Lisbon, were selected in a prospective study from June 2011 to September 2013. For each animal, the following clinicopathological features were recorded: age, breed, reproductive status, progestogens administration, number, location and size of tumor lesions, performed treatment (none, surgery, surgery plus chemotherapy), histopathological classification, malignancy grade, presence of tumor necrosis, lymphatic invasion by tumor cells, lymphocytic infiltration, cutaneous ulceration, regional lymph node involvement, stage of the disease (TNM system) [36], DFS and OS.

Excised mammary glands, mammary tumors and regional lymph nodes from the animals were immediately fixed in 10% formalin neutralized with 0.1 M phosphate buffer (pH 7.2), during a period no longer than 48 hours. All samples were embedded into paraffin blocks and serial histological sections of 3 μm thickness were prepared,

Table 3: HER2 immunohistochemistry scoring criteria

Score	Interpretation
0	No staining
+1	Weak, incomplete membrane staining in any proportion of tumor cells
+2	Complete membrane staining that has either no uniform or is weak in intensity, but with obvious circumferential distribution in at least 10% of cells
+3	Uniform intense membrane staining of at least 10% of invasive tumor cells

Table 4: IHC semi-quantitative scoring system for ER/PR assessment [41, 42]

Proportion of positive staining tumor cells		Average staining intensity	
Score	Interpretation	Score	Interpretation
0	No staining	0	None
1	<1%	1	Weak
2	1-10%	2	Average
3	10-33%	3	Strong
4	33-66%		
5	>66%		
Allred score (0-8) = proportion of positive staining tumor cells (0-5) + average staining intensity (0-3)			

prior to hematoxylin and eosin staining. Carcinomas are classified according to the WHO system adapted by Misdorp et al., 1999 [37] and the degree of malignancy was assessed according to the Elston and Ellis grading system [37, 38], which classifies tumors into grade I (well differentiated), grade II (moderately differentiated), and grade III (poorly differentiated).

Tissue HER2, ER and PR status and Ki-67 assessment

A representative area of each FMC with a diameter of 0.6 cm was selected and tissue sections of 3μm thickness were mounted on glass slides (Star Frost adhesive glass slides, Thermo Scientific, Rockford, USA), deparaffinized with xylene and hydrated in a graded ethanol series to distilled water. For HER2, ER and Ki-67 immunostaining, antigen retrieval was performed by immersing glass tissue slides in citrate buffer (0.01M $NaCH_3COO$, pH 6.0) and using a pressure cooker (2 min at 2 atm), while for PR immunodetection, an immersion in water bath (60 min at 95ºC) was performed [13]. Slides were cooled for 10 min at room temperature and rinsed twice for 5 min in triphosphate buffered saline (TBS). Endogenous peroxidase activity was blocked with Peroxidase Block Novocastra (Novocastra, Newcastle, UK) for 5 min. Tissue samples were then incubated *at 4ºC overnight, in a humidified chamber,* with the following primary antibodies: mouse anti-HER2 (clone CB11, 1:200, Invitrogen, Carlsbad, CA, USA), mouse anti-ER (clone 6F11, 1:125, Thermo Scientific), rabbit anti-PR (clone 1E2, ready-to-use, Ventana, Tucson, USA) and rabbit anti-Ki-67 (polyclonal, 1:500, Thermo Scientific). The staining was performed using a modified streptavidin-peroxidase conjugate method based on the poly-HRP anti-rabbit IgG detection system (Novolink MaxPolymer Detection System, Leica Biosystems, Wetzlar, Germany), following the manufacturer's guidelines. Finally, tissue sections were counterstained with Mayer's hematoxylin (Merck, New Jersey, USA). HER2 immunoreactivity was scored according to the American Society of Clinical Oncology's recommendations [19], as summarized in Table 3. Briefly, FMC were classified as HER2-negative when scored 0 or +1 and HER2-positive if scored as +2 or +3 [13, 39]. Mammary carcinomas were also evaluated for ER/PR status using the Allred score system on a scale of 0 to 8 (Table 4), and only tumors with a score ≥ 2 were considered positive [40-42]. The Ki-67 proliferation index was determined by dividing the number of tumoral cells showing positive nuclear immunostaining per 1000 tumor cells analyzed over at least three high-amplified microscopic fields [43]. Tumors were considered highly proliferative when more than 14% of the neoplastic cells nuclei expressed Ki-67 [43, 44].

Finally, histological samples of feline mammary carcinomas with previous known ER/PR/HER2 status were used as controls, whereas a feline tonsil tissue sample was used as a positive control for the assessment of Ki-67 index, according to the manufacturer's instructions.

All slides were independently subjected to blind scoring by two independent pathologists. Discordant interpretations were further debated and settled using a multiobserver microscope.

Quantitative immunoassays to measure sHER2 levels

A blood sample was collected from all the sixty queens with mammary carcinoma and from twenty healthy queens presented for elective ovariohysterectomy. The serum was separated from clotted blood by centrifugation (1500 g, 10 min, 4°C), stocked in small aliquots (100 µL) and stored at -80°C.

Considering the extensive sequence homology between human HER2 receptor and its homologue in *Felis catus* (93%), sHER2 levels in cats were evaluated by using a commercial ELISA-based kit, suitable to measure sHER2 levels in humans, and also by an optimized Dot blot assay procedure. Measurements were performed in a blind manner, without knowing IHC results and discarding all feline blood samples that show hemolysis (n = 13), as recommended for humans [45].

Enzyme-linked immunosorbent assay (ELISA)

Serum HER2 levels were measured in 67 cats (47 ill and 20 healthy) using an ELISA sandwich assay (sHER2 Platinum ELISA kit, eBioscience, San Diego, USA), following the manufacturer's recommendations. A standard curve was generated using seven solutions of recombinant human HER2-ECD (rHER2-ECD) with known concentrations (0.16, 0.31, 0.63, 1.25, 2.5, 5 and 10 ng/ml). Briefly, first row of a 96-well ELISA plate was coated with 100 µl/well of each rHER2-ECD dilution, in duplicate, on "standards wells", whereas 10 µl of each serum sample was added to 90 µl of assay buffer in "sample wells", also in duplicates. After two consecutive washes (2×300µl with Wash Buffer), 50 µl of an HRP-conjugated mouse anti-IgG was added to each well and incubated at 37°C, for 2 hours, on a microplate shaker at 100 rpm. After a second washing step (3×300 µl), 100 µl of the 3,3′,5,5′-tetramethyl-benzidine (TMB) substrate solution was added to each well and the final mixture was incubated at RT, for 10 min, in the dark. The reaction was interrupted by adding 100 µl of stop solution per well and absorbance was measured by a spectrophotometer (LabSystems IEMS Reader MF, Labsystems/Thermo Scientific, Helsinki, Finland) using 450 nm as the primary wavelength and 620 nm as reference wavelength.

Dot blot assay

After an initial optimization using serum samples from healthy cats, two FMC cell lines with known HER2 expression status and three antibodies against HER2, detection and quantification of sHER2 levels were performed in the sera of 67 cats. Briefly, after drawing a 1 cm square grid on a nitrocellulose membrane (Protan - BA83, Whatman GmbH, Dassel, Germany) with a pencil, 1 µl of each serum sample was spotted at the center of each square, allowing the evaluation of 40 sera per membrane (8 cm x 6 cm). The membranes were then dried at 37ºC for an hour, before being washed and blocked with 0.1M Tris-buffered saline buffer containing 0.1% Tween 20 and 1% bovine serum albumin (TBST-BSA, pH 8.0), for 30 min at room temperature (RT). Then, blots were incubated during 90 min at RT, with an anti-HER2 antibody (clone SP3, dilution 1:1000, Zytomed, Berlin, Germany) that specifically recognizes the extracellular domain of HER2 [46, 47]. Later, membranes were washed for 30 min with TBST (3 x 10 min) and incubated for 30 min at RT with an appropriate secondary antibody (1:100,000; goat anti-rabbit IgG horseradish peroxidase conjugated - HRP, Southern Biotech, Birmingham, USA). The presence of HER2 in serum was revealed by the use of an enhanced chemiluminescence detection kit (Clarity Western ECL Substrate, Bio-rad, California, USA), and a semi-quantitative analysis was performed by comparing the signal intensity obtained in serum dots to the signal intensities of crescent concentrations of rHER2-ECD (5, 10, 15, 25, 50, 75 and 100 ng/ml, eBioscience, San Diego, USA) diluted in 30% BSA-Tris-buffered saline plus 0.05% Tween 20 (TBST) and spotted in the first line of the nitrocellulose membrane. Serum HER2 levels were scored by two independent observers and, discordant interpretations were discussed to reach a consensus. All serum samples were evaluated in three independent experiments.

Finally, two antibodies raised against different regions of the intracellular domain of HER2 receptor were also used to determine whether this domain is present in the soluble HER2 forms, thus detectable in the serum of cats (clone A0485, dilution 1:3500, Dako Denmark A/S, Glostrup, Denmark and clone 4B5, dilution 1:20, Ventana Medical Systems Inc., Tucson, AZ, USA).

Western blotting analysis

To evaluate antibody specificity, two feline serum samples with distinct HER2 levels previously determined by the Dot blot assay, were probed with the anti-HER2 monoclonal antibody raised against the ECD (clone SP3). The molecular weight of the sHER2 fragments were compared with the band of recombinant human HER2-ECD, and with the bands detected in whole cell extracts of a human HER2-overexpressing breast cancer cell line (SKBR3, ATCC, Manassas, Virginia, USA) and a feline HER2-positive mammary carcinoma cell line (FMCp, kindly provided by Prof. Nobuo Sasaki, Tokyo University, Japan).

SKBR3 and FMCp cells grown in 30mm dishes were washed three times with phosphate-buffered saline (PBS) and lysed in radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris, pH 8.2; 150 mM NaCl; 0,5% NP40;

0,5% sodium deoxycolate; 0,1% SDS) supplemented with the following protease and phosphatase inhibitors: cOmplete, Mini, EDTA-free (Roche), Phosphatase Inhibitor Cocktail Set V, 50x (Calbiochem, San Diego, USA) and PhoStop (Roche, Basel, Switzerland). After lysis, whole cell extracts were boiled at 95ºC for 15 min, centrifuged at 14,000 g for 2 min at 4ºC and stored at -80ºC.

The diluted rHER2-ECD, the whole cell lysates and the serum samples were fractioned by 7.5% SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane with 0.2 μm pore diameter (Whatman Schleicher & Schuell, Whatman GmbH, Dassel, Germany). The immunoblot was initially blocked using 2.5% (w/v) bovine serum albumin (BSA, Sigma) in TBST, to inhibit non-specific binding. For immunoblot analysis, membranes were then incubated with the primary and secondary antibodies used in the Dot blot assay. Signal intensity of reactive bands was detected by autoradiography using an enhanced chemiluminescence detection kit (Luminata Crescendo, ECL detection system, Millipore, Darmstadt, Germany).

Statistical analysis

All statistical analyses were carried out using the Statistical Package for the Social Sciences for Windows software (SPSS, version 21.0, IBM, Armonk, New York, USA) and a two-tailed *p* value less than 0.05 was considered statistically significant.

After testing for normality, the Wilcoxon test was used to compare the sHER2 levels between cats with mammary carcinoma and healthy ones, and also to compare sHER2 levels and tissue HER2 status among cancer group cats. Receiver-operating characteristics (ROC) curves were performed to choose the optimal cutoff value for the ELISA and Dot blot assay, and to determine the sensitivity and specificity of both techniques using IHC as a gold standard technique. The concordance between the three assays was estimated by the Kappa test and correlations were evaluated by Spearman's rank correlation coefficient. The Fisher's exact test was used to assess the associations between sHER2 levels/ tumor HER2 status and the clinicopathological features (breed, reproductive status, progestogens administration, prescribed treatment, number and location of tumor lesions, primary tumor size, lymph node status, stage of disease, histopathological classification, malignancy grade, presence of necrotic areas within the neoplasia, lymphatic vessel invasion by tumor cells, lymphocytic infiltration, cutaneous ulceration, Ki-67 index, HER2, ER and PR status). Whenever a cat showed multiple mammary tumors, the carcinoma with higher HER2 score (assessed by IHC), was the one selected for further studies. In cats with two or more mammary carcinomas showing equal HER2 scores, the lesion with higher malignancy grade and size was selected for statistical studies, since these two features have previously been associated with poor prognosis [48, 49]. Animals were also grouped by the tumor size, according to the TNM classification (< 2 cm; 2-3 cm; > 3 cm).

Overall survival (OS) period was defined as the time elapsed between the initial diagnosis and the death/ euthanasia due to tumor metastasis. Disease-free survival (DFS) time was calculated from the date of surgery to the date of relapse (local, in other mammary gland or in distant organs) or death from cancer-related causes. Survival curves were estimated using the Kaplan-Meier method and the Log-rank test was used to compare the outcome (OS median and DFS median), regarding sHER2 levels and tumor HER2 status. For OS analysis, animals that died from a disease unrelated to mammary tumors or were lost during the follow-up were excluded.

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CONFLICTS OF INTERESTS

The authors declared no potential conflicts of interest with respect to research, authorship, and/or publication of this article.

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