

Review

# Emerging Biomarkers and Targeted Therapies in Feline Mammary Carcinoma

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**Abstract:** Feline mammary carcinoma (FMC) is a common aggressive malignancy with a low survival rate that lacks viable therapeutic options beyond mastectomy. Recently, increasing efforts have been made to understand the molecular mechanisms underlying FMC development, using the knowledge gained from studies on human breast cancer to discover new diagnostic and prognostic biomarkers, thus reinforcing the utility of the cat as a cancer model. In this article, we review the current knowledge on FMC pathogenesis, biomarkers, and prognosis factors and offer new insights into novel therapeutic options for HER2-positive and triple-negative FMC subtypes.

**Keywords:** feline mammary carcinoma; biomarkers; feline *her2* mutations; targeted therapies; comparative oncology model



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## 1. Introduction

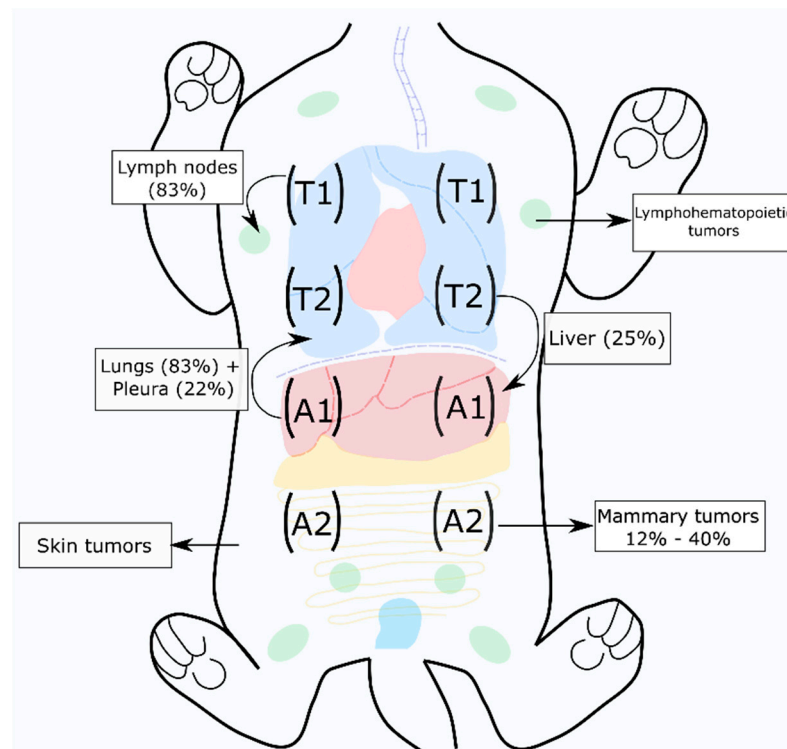
Cats are the most popular companion animals in developed countries, outnumbering dogs [1]. As they share similar environmental conditions with their owners, as well as genetic and biological features, cats have been used as models for human ophthalmic diseases, type 2 diabetes and, since the full sequencing of their genome, comparative oncology studies [2–5]. Cats are also emerging as promising animal models for preclinical testing of HER2-positive and triple-negative mammary carcinoma therapies [6–11]. Feline mammary carcinoma (FMC) is the third most common type of cancer in cats, corresponding to 17% of all tumors in queens, and is usually malignant [12], as is human breast cancer (HBC) [13], occurring in 90% of the cases due to somatic mutations [14] and showing comparable risk factors. It is the first cause of death in cats, with short overall survival (OS), and very poor prognosis, as it tends to be diagnosed at late stages and has limited therapeutic options that show weak responses [4,15]. FMC has similar anatomical, biological and clinical features to HBC, although metastatic mechanisms remain poorly understood [4], and is likewise classified in different molecular subtypes: luminal A, luminal B, epidermal growth factor receptor 2-positive (HER2-positive) and triple-negative normal-like and basal-like [16,17].

Using the extensive knowledge available on HBC, it is possible to find comparable diagnostic and prognostic biomarkers, as well as therapeutic targets, like the HER2 protein, that may improve FMC's prognosis. These epidermal growth factor receptor (EGFR) family members are commonly targeted in breast cancer therapies by antibodies and/or small inhibitors that disrupt different cellular pathways [18–24]. Other emerging agents that have already proved valuable in FMC in vitro studies [9] include histone deacetylase inhibitors (HDACi) [25,26], and microtubules inhibitors (MTi) [27–29].

This review summarizes the similarities between FMC and HBC, with special emphasis on the progress attained in FMC, in particular towards better understanding of its clinical hallmarks and molecular and biological features. Furthermore, the antiproliferative effects of several compounds already approved for HBC therapy are discussed in the context of FMC cell-based models as future treatments proposed for cats with mammary carcinoma.

## 2. Feline Mammary Carcinoma

FMC is a common disease in middle-aged to old queens (10 to 12 years) [30,31], more frequent in the Siamese and domestic short-hair breeds, with an OS time of around 1 year [16,17,31,32]. It occurs more frequently in unspayed cats, being associated with the expression of estrogens (ER) and progesterone (PR), and hormonal therapy [33]. Indeed, an ovariectomy before six months of age is known to be a protective factor, reducing FMC development in 91% of cases [12,33]. Mammary tumors are usually malignant (80 to 90%), occurring with higher frequency in the abdominal glands and in 50% to 90% of the cases leading to metastasis [31], most commonly in the regional lymph nodes and lungs [12] (Figure 1).



**Figure 1.** Mammary carcinoma is the third most common tumor in cats, with a high metastasis rate, frequently to lymph nodes and lungs [17,31]. The black arrows indicate the most frequent tumor locations and metastasis pattern.

At the time of diagnosis, identification of multiple masses is common, usually in the same mammary chain, whereas in women, a single mass is observed in most cases. The same anatomic classification (in situ vs. infiltrative) and histologic grade [4] are reported for FMC and HBC. Thus, mammary tumors are defined as simple or complex, with secretory and ductal cells documented, and identified as inflammatory disease, when less differentiated cells and lymphatic-dermic obstruction are present.

### 2.1. Mammary Tumor Diagnosis and Classification

Early-stage mammary tumors present as mobile, palpable, discrete masses. However, as tumor diagnosis is usually belated, patients tend to present several masses, with ulceration (25% of the cases) and necrosis. The physical exam may also reveal edema, exudate in the nipples, and a decrease in the temperature of the pelvic region. For correct diagnosis and prognosis before surgery, a precise tumor classification is mandatory. Even though cytology is easy to perform, most of the time, results are inconclusive [12], making biopsy crucial to confirm tumor stage and malignancy grade [31].

Although a standardized classification system does not exist, the same parameters used for HBC are applied (Table 1), with TNM (Tumor, Nodes and Metastasis) being the most widely used staging system [34,35]. Tumor classification also considers the malignancy grade, which takes into account tumor size, tissue invasion, ulceration, lymphovascular invasion, and lymph node status [35]. Additionally, a histopathological analysis is advised, with higher frequencies of adenocarcinomas in situ, tubulopapillary, solid, or cribriform masses reported. Concerning histologic grade, the Elston and Ellis (EE) Grading System is usually employed, with the majority of tumors defined as moderate to less differentiated masses [31,36]. Moreover, the molecular characterization in luminal A, luminal B, HER2-positive and triple-negative subtypes [17], as in women, reveals itself as an important prognostic factor, and may unveil targets for a directed therapy.

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

Tumor Classification of Feline Mammary Carcinomas			
Tumor Clinical Stage *			
Stage	Tumor size (T)	Lymph node status (N)	Metastasis (M)
1	T1 (<2 cm)	N0	M0
2	T2 (2–3 cm)	N0	M0
3	T1	N1	M0
	T2	N1	M0
4	T3 (>3 cm)	N0/N1	M0
	Any	N0/N1	M1
Histological Grade (EE System)			
Histologic feature		Score	
Tubule formation			
>75%	1		
10–75%	2		
<10%	3		
Nuclear pleomorphism		Sum of the scores	Grade
Mild	1	3–5	I
Moderate	2	6–7	II
Marked	3	8–9	III
Mitotic count (per 10 microscopic fields)			
0–5	1		
6–10	2		
>11	3		

\* 0—indicates absence of the characteristic; 1—indicates presence of the characteristic.

## 2.2. Feline *her2* Mutations Could Be Associated with Tumor Development

Chromosomal instability is a key factor for tumor development. Concerning the HER2-positive subtype, which presents similar clinicopathological features to HBC [17] and is one of the most common in the cat (33% to 60% of all cases) [17,37], a deep analysis of the *her2* gene, by comparison to the human counterpart may be considered, as they share a 90% to 95% homology [4,38].

HER2 is a glycoprotein that contributes to cell proliferation, differentiation, and survival [39,40]. Interestingly, in women, breast cancer progression may be associated with HER2 amplification, conditioned by a gain in *her2* gene copy numbers, observed by in situ hybridization [41–43]. By comparison, a different process occurs in the cat, with an increase in *her2* mRNA copy numbers, evaluated by real-time reverse transcriptase (RT)-qPCR [44–46].

We know that in breast cancer patients, *her2* is mutated in 2 to 3% of primary tumors, the most common mutations occurring in the HER2-negative breast cancer subtype, which has a reported rate of incidence of 70% in the cat [10,47]. In these species, 90% of the breast tumors also have acquired somatic mutations [14], mostly occurring in the TK domain [10,11,14,46], with two single variants (SV) and two haplotypes described [38]. The observed *her2* mutations are suggestive of an association with the clinicopathological features, being correlated with primary tumor size and the number of tumor masses [10,38]. Furthermore, SVs at splicing regions, *her2* polymorphisms, or mutations in introns may be originating different isoforms of the protein, triggering the HER2 activity and tumor aggressiveness [38] or therapy resistance [48,49], as has already been described in HBC patients. Considering the *her2* gene sequence that encodes for part of the HER2 protein's extracellular domain, three non-synonymous genomic variants were reported, predicting an alteration of the 3D structure of the protein by computational analysis and modelling [14].

### 2.3. Prognostic Factors for Feline Mammary Carcinoma

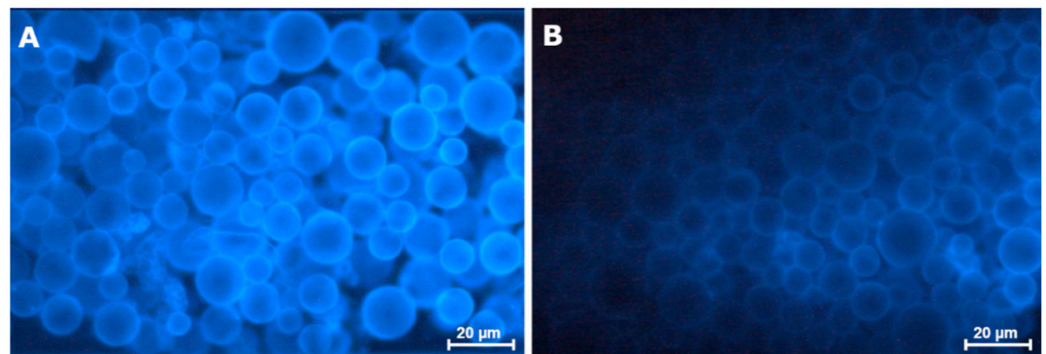
To uncover diagnostic and prognostic biomarkers, as well as new therapeutic targets in cats, the study of the tumor microenvironment, its molecular characterization, and the analysis of systemic alterations is crucial.

In a macroscopic analysis, tumor size is one of the most important prognostic factors in FMC, with masses larger than 3 cm presenting a poor prognosis [36], and conditioning a more aggressive surgical approach [50]. Furthermore, the tumor's histologic grade, presence of lymphatic metastasis and/or lymphovascular invasion [12], as well as tumor stage [51] and subtype [36], have shown to be highly correlated with OS time.

Despite this being a relatively recent field of study, several biomarkers have already been identified that may be involved in FMC prognosis. Molecular expression of Ki-67, evaluated by immunohistochemistry [12], reveals that an index above 14% is associated with poor prognosis [52]. AKT expression, which is usually associated with PR-/ER-negative invasive carcinomas, also correlates with malignancy and non-tumor differentiation, lowering the disease-free survival (DFS) ratio [53]. In parallel, cats with a triple-negative subtype present a higher mTOR expression, as has been described in women [54], this being associated with cancer invasion and metastasis [55]. Moreover, mutations in the *p53* gene involved in cell cycle regulation and tumor suppression have been reported in 18.9% of FMCs [56,57]. Furthermore, overexpression of several molecular biomarkers is also associated with poor prognosis, e.g., macrophage-stimulating protein receptor (RON), related to tumor invasion, cyclo-oxygenase (COX)-2, expressed in malignant FMCs, and topoisomerase II $\beta$  binding protein 1 (TopBP1), which is similar to BRAC2 in HBC [31,58]. Interestingly, the CXCR4/CXCL12 axis, which controls cell survival, migration and proliferation, is also a key factor in feline breast cancer progression and metastasis, as reported for HBC [59,60], and its disruption is associated with lower OS time [60–62]. Additionally, CXCL12 has been reported as a blood serum marker in the cat, particularly for HER2-positive tumors [62]. Finally, analysis of the vascular endothelial growth factor (VEGF) status, shows that this molecule is overexpressed in more aggressive carcinomas [50,63], playing an important role in tumor-associated angiogenesis.

In parallel, an association is reported between the expression of some of these proteins in serum and in the tumor microenvironment, suggesting that serum samples may be used as a non-invasive method for the assessment of checkpoint molecules [64–66]. Interestingly, in the cat, HER2 serum expression is elevated in malignant lesions, lowering OS [17,31], a fact that makes it a promising diagnostic tool. In fact, our group has already shown that a rapid diagnostic kit for the identification of HER2-positive mammary carcinoma through detection of serum HER2 expression levels can be produced. These preliminary experiments, using nanoparticles coated with anti-HER2 fluorescent antibodies, showed that serum HER2 expression levels can be quantified in cats with mammary carcinoma (Figure 2A), by comparison with a control sample (Figure 2B; data not published). Never-

theless, more work is needed in order to define cut-off values, sensitivity and specificity of the test.



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

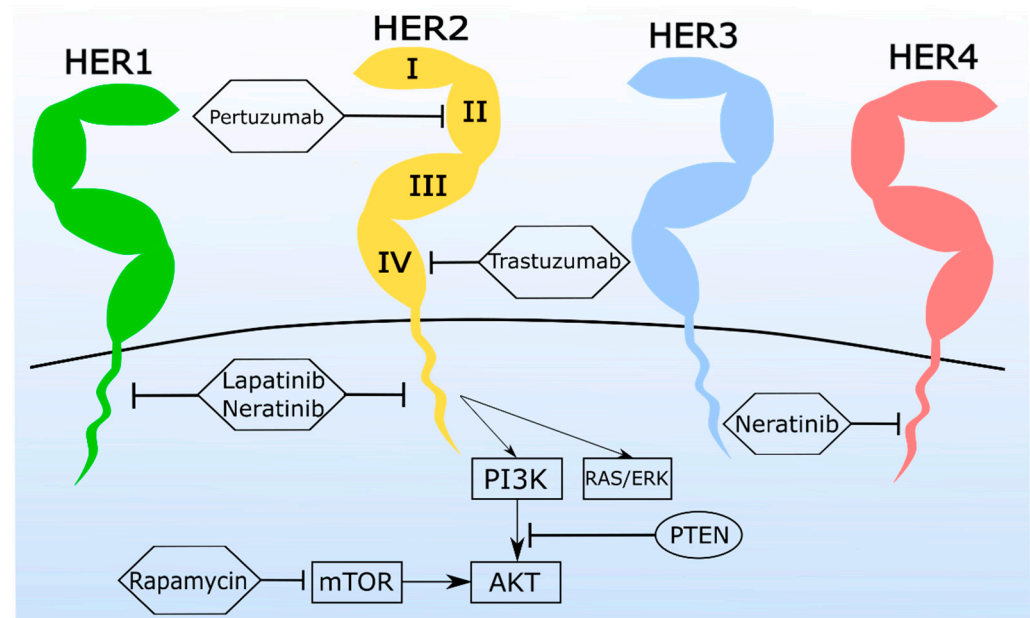
It's widely acknowledged that in women, a chronic inflammatory status, such as that induced by obesity, can be a trigger for mammary tumor development [67,68]. Interestingly, cats with mammary carcinoma present a decrease in the serum leptin/leptin receptor (ObR) ratio [66,69], as has been documented for pre-menopausal women with breast cancer [70]. Furthermore, in animals with FMC, the higher leptin levels are associated with a triple-negative tumor subtype, also as reported for HBC [66,71,72]. In parallel, ObR is associated with an immunosuppressive status [66,73,74], observed in both breast cancer patients [75,76] and cats with mammary carcinoma, and is additionally correlated with the overexpression of cytotoxic T-lymphocyte associated protein 4 (CTLA-4), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) [64,77], and programmed cell death (PD-1)/programmed death ligand-1 (PD-L1) [64,78] in the most aggressive tumor subtypes (HER2-positive and triple-negative) [64,79].

### 3. Feline Mammary Carcinoma Cell-Based Models for Targeted Therapies

In cats, therapeutic options are scarce, the most common being uni/bilateral radical mastectomy, alone, or in combination with chemotherapeutic adjuvant protocols when the Ki-67 index is above 14% [52], which increases the cat's DFS but not OS, due to the high metastasis rate [12,31,80]. Moreover, the agents used tend to have limited efficacy and severe side effects [4,15]. Combination therapy protocols with doxorubicin and cyclophosphamide/carboplatin, for example, show poor response in metastasis [31,81], and tamoxifen shows no significant response [4], as FMC is more commonly ER-negative, unlike HBC [4,33].

Thus, a deep understanding is needed to unveil alternative therapeutic options aimed at improving the cat's clinical outcome. Such studies are limited, however, by a lack of feline cell lines available for cytotoxicity assays, with only 8 having been reported so far [82] and a shortage of in vivo models for preclinical trials, although four FMC xenograft models were recently reported (preliminary report) [83], revealing to be of extreme importance in order to understand the mammary carcinoma biology, development and metastization process [84,85], as the use of nude mouse models [84]. Here we review some therapeutic drugs (Figure 3) approved for HBC therapy that were recently tested in FMC cell-based models (Table 2; CAT-MT from the European Collection of Authenticated Cell Culture, England; FMCp and FMCm kindly provided by Prof. Nobuo Sasaki and Prof. Takayuki Nakagawa, University of Tokyo, Japan). The reported results represent an initial step towards the development of more effective therapeutic options for cats with

mammary carcinoma and interestingly, all assays reveal promising results and a conserved mechanism of action [10,11], by comparison to a human HER2-overexpressing cell line (SKBR-3; American Type Culture Collection [86]).



**Figure 3.** The HER2 pathway is a common target in human breast cancer therapy, revealing promising results for the treatment of cats with mammary tumors.

**Table 2.** Classification and molecular characterization of FMC cell lines [10,11].

Cell Line	Tumor Classification	ER (%)	PR (%)	Ki-67 (%)	Ck5/6 (%)	HER2
CAT-M	Mammary Adenocarcinoma	10	80	50.2	<1	2+
FMCp	Primary breast tumor	60	Negative	57.4	<1	0
FMCm	Metastatic lymph node	2	Negative	68.5	<1	1+

ER—Estrogen Receptor; PR—Progesterone Receptor; Ck5/6 and HER2 [17]; Ki-67 index [52].

More *in vitro* studies are needed, however, to fully characterize the effect of the anti-tumor compounds tested, as well as develop proper xenograft models for preclinical studies.

Furthermore, despite the real value of FMC xenograft models, some limitations could be pointed out, such as the need of induced tumors, absence of a competent immune system, or comparable pharmacokinetic and pharmacodynamics responses, when compared to other mammals [87]. Thus, the use of the cat as an *in vivo* oncology model for HBC reveal several advantages to take into consideration, representing epidemiologic, clinical and morphologic similarities with its human counterpart [88].

### 3.1. Monoclonal Antibodies (mAbs) and Antibody-Drug Conjugates (ADC) Are a Promising Tool for the Treatment of Feline Mammary Carcinoma

The HER2 protein is a common target for molecular therapy in HBC patients, using mAbs that interact by shape complementarity [18], thus preventing HER2 dimerization and activation of its downstream pathways [89]. These compounds are a good alternative to Tyrosine Kinase inhibitors (TKi), which are toxic for the majority of tissues, showing severe side effects [90]. Recent studies have revealed a 93% similarity between human and feline HER2 [6,14] (*homo sapiens*, UniProt P04626; and *felis catus*, UniProt H9BB15), which allowed for testing of humanized-mAbs against FMC (Table 3).

**Table 3.** Monoclonal antibodies (pertuzumab and trastuzumab) and antibody-drug conjugate (T-DM1) compound induce cell apoptosis, with promising antiproliferative effects in FMC in vitro models [11].

mAb	Target	Mechanism of Action	Breast Cancer Clinical Application	References	FMC In Vitro System			
					Cell Line	HER2 Status	Concentration ( $\mu\text{g}/\text{mL}$ )	Cytotoxicity (%)
Pertuzumab	HER2 ECD II	Prevents HER2 heterodimerization; Inhibits EGFR downstream pathways; Stimulates ADCC and apoptosis	HER2-overexpressing and metastatic tumors	Agus et al., 2002 [91]; Scheuer et al., 2009 [92]; Baselga et al., 2010 [93]; Metzger-Filho et al., 2013 [94]; Richard et al., 2016 [22]; and Yamashita-Kashima et al., 2017 [89]	CAT-M	2+	10,000 ( $\text{EC}_{50} = 2837.92 \mu\text{g}/\text{mL} \pm 1.50$ )	60.2
					FMCp	0	10,000 ( $\text{EC}_{50} = 928.97 \mu\text{g}/\text{mL} \pm 1.11$ )	52.1
					FMCm	1+	10,000 ( $\text{EC}_{50} = 1205.04 \mu\text{g}/\text{mL} \pm 1.23$ )	61.8
Trastuzumab	HER2 ECD IV	Prevents HER2 homodimerization; Block receptor internalization and degradation; Prevents HER2 shedding; Induces ADCC and apoptosis	HER2-overexpressing invasive, metastatic and early-stage tumors	Klapper et al., 2000 [95]; Cho et al., 2003 [18]; J. Piccart-Gebhart, 2005 [96]; Nahta et al., 2007 [97]; D. Slamon, 2011 [98]; Menyhart et al., 2015 [99]; Richard et al., 2016 [22]; and Kast et al., 2017 [100]	CAT-M	2+	10,000 ( $\text{EC}_{50} = 3047.89 \mu\text{g}/\text{mL} \pm 1.43$ )	92.6
					FMCp	0	10,000 ( $\text{EC}_{50} = 3243.40 \mu\text{g}/\text{mL} \pm 2.29$ )	60.1
					FMCm	1+	10,000 ( $\text{EC}_{50} = 528.45 \mu\text{g}/\text{mL} \pm 1.14$ )	82.7
T-DM1	HER2 ECD II; CKAP5	Prevents HER2 homodimerization; Inhibits microtubule assembly; Induces cell apoptosis	HER2-positive, advanced, early stage and metastatic tumors	Phillips et al., 2008 [19]; Lambert and Chari, 2014 [101]; Von Minckwitz et al., 2019 [24]; Lacasse et al., 2020 [102]; and Liu et al., 2020 [103]	CAT-M	2+	1000 ( $\text{EC}_{50} = 19.63 \mu\text{g}/\text{mL} \pm 1.22$ )	94.0
					FMCp	0	1000 ( $\text{EC}_{50} = 88.72 \mu\text{g}/\text{mL} \pm 1.29$ )	74.2
					FMCm	1+	1000 ( $\text{EC}_{50} = 52.84 \mu\text{g}/\text{mL} \pm 1.50$ )	53.8

In the assays testing both mAbs (pertuzumab and trastuzumab), a dose-dependent antiproliferative effect, as well as a conserved cell death mechanism by apoptosis, were demonstrated, even though feline cell lines present lower HER2 expression levels when compared to the human SkBR-3 cell line [11].

In the pertuzumab assay, the addition of heregulin to the cell medium was suggested, which would allow for mAb-HER2 heterodimerization [104,105], thus improving cytotoxicity results. Furthermore, an antiproliferative effect was described in the FMCp HER2-negative cell line [11,53,55]. In fact, pertuzumab has already been suggested for the treatment of triple-negative HBC expressing HER2-103, a recently described protein encoded by a circular form of the HER2 gene that is associated with worse overall prognosis for these patients [106]. A pertuzumab-HER3 interaction has also been reported in human lung cancer [107]. This suggests there may be a real benefit of pertuzumab in the treatment of HER2-negative FMC. However, more studies are needed. In parallel, testing of trastuzumab on the same FMCp cell line also revealed a promising antiproliferative effect. Despite the lower cytotoxic response, due to a lack of HER2 expression, this result proposes that cats with HER2-negative tumors may benefit from the use of trastuzumab, as suggested for human triple-negative breast cancer that expresses an activated form of HER2 (HER2<sup>Y877</sup>) [108].

Other compounds used for the treatment of breast cancer are the ADCs, e.g., trastuzumab-emtansine (T-DM1). This ADC allows a targeted delivery of the cytotoxic agent, DM-1 a microtubule inhibitor, to HER2-overexpressing tumor cells, decreasing its side effects [101]. Testing of T-DM1 in FMC cell-based models resulted in promising cytotoxic effects, leading to a conserved cell death mechanism by apoptosis. Interestingly, for the HER2-negative FMCp cell line a high cytotoxic effect was observed [11], which could be explained by the interaction of DM1 with the cytoskeleton-associated protein 5 (CKAP5), a microtubule assembly regulator, as described in human HER2-negative cells [109]. More studies are needed to evaluate the expression status of the CKAP5 protein in triple-negative FMC and be in a better position to propose T-DM1 for the treatment of feline HER2-negative breast cancer.

Despite the good results of these cytotoxicity assays, a 3D cell culture system is needed for correct prediction of receptor-mAb conformational interactions [110,111], and proper feline mAbs should be designed.

### *3.2. Tyrosine Kinase Inhibitors (TKi) Are Valuable in Feline Mammary Carcinoma Therapy*

TKis are small chemical compounds that prevent protein phosphorylation, by interacting with the cytoplasmic catalytic kinase domain [21], for example, of EGFR family members. These compounds block HER2 signaling for cell proliferation via the RAS-ERK pathway [112] and for cell death inhibition via the PI3K-AKT-mTOR pathway [113]. Despite their side effects, they are a suitable alternative for patients that show resistance to anti-HER2 mAbs, which in women with HER2-positive breast cancer is around 50% [21,114]. TKis (lapatinib and neratinib) have been tested against FMC in in vitro models, with promising cytotoxic effects obtained (Table 4).



**Table 4.** Tyrosine kinase inhibitors (lapatinib and neratinib) presented valuable cytotoxic effects in the FMC in vitro models, suggesting a conserved mechanism of action [10].

TKi	Target	Mechanism of Action	Breast Cancer Clinical Application	References	FMC In Vitro System			
					Cell Line	HER2 Status	Concentration (nM)	Cytotoxicity (%)
Lapatinib	HER1 and HER2	Reversible; Prevents EGFR family members phosphorylation	Solid, advanced and metastatic HER2-positive tumors; Valuable in combined protocols	Frenel et al., 2009 [115]; Opdam et al., 2012 [116]; Shi et al., 2016 [117]; and Stanley et al., 2017 [118]	CAT-M	2+	50,000 (IC <sub>50</sub> = 3930 nM ± 49)	100
					FMCp	0	50,000 (IC <sub>50</sub> = 4870 nM ± 100)	100
					FMCm	1+	100 × 10 <sup>3</sup> (IC <sub>50</sub> = 17,470 nM ± 100)	100
Neratinib	HER1; HER2 and HER4	Irreversible; Prevents EGFR family members phosphorylation; Surpass lapatinib resistance	Adjuvant treatment of HER2-positive early-stage and metastatic breast cancer	Tiwari et al., 2015 [119]; Sun et al., 2015 [40]; Cocco et al., 2018 [23]; and Food and Drug Administration (FDA)	CAT-M	2+	25	33.5
					FMCp	0	250	79.4
					FMCm	1+	1000	31.4

The lapatinib exposure assay demonstrated a dose-dependent antiproliferative effect with a conserved mechanism of action, by reducing HER1 (Y1173) and HER2 (Y1221+Y1222) phosphorylation patterns, and their downstream pathways, AKT (S473) and ERK 1/2 (T202/Y204+T185/Y187), involved in cell cycle progression and apoptosis [10,120,121]. Interestingly, like in the feline HER2-positive cell lines tested (CAT-M and FMCm), the feline HER2-negative cell line (FMCp) presented a 100% cytotoxic response [10]. These results suggest an interaction between lapatinib and HER1, which is an EGFR family member usually upregulated in women with triple-negative breast tumors [122,123]. Moreover, lapatinib is described as activating NF- $\kappa$ B in triple-negative HBC, inducing cell apoptosis [124,125], a different pathway that should be investigated in cats. This study also showed that lapatinib induces the accumulation of membrane HER2 [10,125], suggesting protein stabilization by the inhibition of HER2 phosphorylation and prevention of receptor ubiquitination [126], as described for human cells.

In parallel, neratinib assay revealed similar antiproliferative effects in all feline cell lines tested, including the FMCp HER2-negative cell line [10], which may suggest an interaction with other EGFR family members, such as HER1 [122,123], or HER4 [127,128]. In contrast, a dose-dependent effect was not observed in the FMCm metastatic cell line [10], suggesting a resistance pattern, as has been documented in humans, e.g., because of increased activity of the cytochrome P4503A4 [129], or overexpression of NmU, a protein involved in breast cancer progression and metastasis [130].

At this point, the need for an *in vivo* system arises to characterize the cats' systemic response to these compounds.

### *3.3. Combination Therapy Shows Synergistic Antiproliferative Effects in Feline Mammary Carcinoma Cell Lines*

Acquired resistance to therapy is a well-documented phenomena in women, and in order to surpass this and improve patients' clinical outcome, combined therapies have become a valuable tool [131]. Different combinations are found in the literature, e.g., of different mAbs [22,131,132], of mAbs with TKis [133–135], and of TKis with the mTOR inhibitor (mTORi) rapamycin [136,137]. In this way, we are able to block different cell proliferation and survival signaling pathways [6,32,40,54] using lower drug concentrations (Table 5), and this is a strategy that could become important in cats.

It is known that pertuzumab is complementary to trastuzumab in HBC mAb combined therapy [92], presenting a synergistic antiproliferative effect, and this same effect has been observed in FMC cell lines [11].

Combination therapy with the mAb pertuzumab and the TKi, lapatinib, also shows a synergistic effect in FMC cell-based models. This effect is particularly noticeable in the FMCp cell line [11], as the combined drugs are able to target different EGFR family members [106]. This combination was effective in the metastatic FMCm cell line as well [11], a promising result, as it has already been approved in humans for metastatic tumors [115,116]. Trastuzumab also acts synergistically with lapatinib revealing an additive antiproliferative effect in FMC cell lines [11]. In fact, this protocol has already proved effective against HBC, particularly for HER2-positive and metastatic therapy, improving patients DFS [126,138].

In conjugation protocols between TKis and rapamycin it is important to characterize mTORi effects. The mTOR pathway [139,140] is the target of rapamycin in adjuvant protocols. This compound presents immunosuppressant anticancer properties, but with no effective cytotoxic response when used as a single agent [10], as described for human cancers [54]. Interestingly, in the FMCp HER2-negative cell line, good results were obtained, which could be explained by mTOR overexpression, something that has been reported in cats with HER2-negative mammary carcinomas [55] and also breast cancer patients [141]. Its conjugation with lapatinib, however, reveals a synergistic antiproliferative response in all feline cell lines [10]. Thus, it may be described as a valuable tool in combined protocols, namely for metastatic breast cancer therapy [10,115,116]. In parallel, conjugation with neratinib also reveals synergistic antiproliferative effects, particularly noticeable in the

FMCm and FMCp cell lines [10], being this protocol recommended for human metastatic HER2-positive and triple-negative breast cancer therapy [142,143].

**Table 5.** Combined protocols present synergistic antiproliferative effects in FMC cell-based models by blocking different HER2 pathways [10,11].

Combined Protocol	Blocked Pathways	FMC In Vitro Assay			
		Cell Line	HER2 Status	Increase in Cell Cytotoxicity (%)	<i>p</i> -Value
<b>mAbs combination</b>					
Pertuzumab plus Trastuzumab	HER2 ECD II and HER2 ECD IV	CAT-M	2+	26.4	0.0018
		FMCp	0	11.7	0.0184
		FMCm	1+	29.5	<0.001
<b>mAb plus TKi</b>					
Pertuzumab plus Lapatinib	HER2 ECD II; HER1 and HER2 TK domain	CAT-M	2+	69.4	<0.001
		FMCp	0	47.5	<0.001
		FMCm	1+	41.5	<0.001
Trastuzumab plus Lapatinib	HER2 ECD IV; HER1 and HER2 TK domain	CAT-M	2+	71.9	<0.001
		FMCp	0	62.0	<0.001
		FMCm	1+	27.2	0.0017
<b>TKi plus mTORi</b>					
Lapatinib plus Rapamycin	HER1 and HER2 TK domain and mTOR complex	CAT-M	2+	51.9	0.0360
		FMCp	0	47.5	<0.001
		FMCm	1+	85.6	<0.001
Neratinib plus Rapamycin	HER1, HER2 and HER4 TK domain and mTOR complex	CAT-M	2+	47.4	0.0044
		FMCp	0	44.1	0.0034
		FMCm	1+	66.7	<0.001

### 3.4. Novel In Vitro Approaches to Feline Mammary Carcinoma Therapy

Current knowledge on HBC reveals different tumor subtypes, e.g., the triple-negative, which has no directed therapy [55], as well as development of therapeutic resistance, which requires different strategies to improve patients clinical outcome. Highlighting the importance of the cat as a model, and since few studies exist [144,145], the antiproliferative effects of new compounds, e.g., HDACi [146] and MTi [147], were recently tested in FMC in vitro models (CAT-M and FMCp), revealing themselves as promising agents for molecular targeted therapy (Table 6).

**Table 6.** Histone deacetylase inhibitors and microtubule inhibitors show promising cytotoxic effects in FMC cell-based models, suggesting a conserved mechanism of action [9].

Class of the Compound	Mechanism of Action	References	Agent	FDA Approval	FMC In Vitro Assays	
					Cell Line	IC <sub>50</sub> Value
HDACi ( $\mu$ M)	Inhibits histone deacetylases leading to chromatin relaxation and uncontrolled gene expression; Induces cell cytotoxicity and death by apoptosis	Xu et al., 2007 [148]; Chun, 2015 [149]; and FDA	CI-994	Experimental	CAT-M FMCp	16.470 $\pm$ 1.904 9.616 $\pm$ 2.150
			Panobinostat	Yes; 2015	CAT-M FMCp	0.042 $\pm$ 0.067 ND #
			SAHA	Yes; 2006	CAT-M FMCp	4.416 $\pm$ 0.453 2.571 $\pm$ 0.578
			SBHA	Experimental	CAT-M FMCp	45.230 $\pm$ 4.692 33.830 $\pm$ 6.454
			Scriptaid	ND	CAT-M FMCp	3.392 $\pm$ 0.403 3.090 $\pm$ 0.691
			Trichostatin A	Experimental	CAT-M FMCp	0.263 $\pm$ 0.062 ND #
MTi (nM)	Inhibits microtubule polymerization, leading to cytoskeleton disruption; Induces cell cycle arrest and apoptosis	Risinger et al., 2015 [27]; Zang et al., 2018 [29]; Steinmetz and Prota, 2018 [28]; and FDA	Colchicine (Destabilizing agent)	Yes; 2009	CAT-M FMCp	1.472 $\pm$ 0.484 5.876 $\pm$ 0.968
			Nocodazol (Destabilizing agent)	Experimental	CAT-M FMCp	12.270 $\pm$ 3.455 30.840 $\pm$ 8.499
			Vinblastine (Destabilizing agent)	Yes; 2011	CAT-M FMCp	0.570 $\pm$ 1.080 6.563 $\pm$ 1.514
			Paclitaxel (Stabilizing agent)	Yes; 2002	CAT-M FMCp	1.939 $\pm$ 1.134 8.646 $\pm$ 2.337

# ND—Not determined.

Histone deacetylases are enzymes that control gene expression, and their dysregulation is associated with tumor development [150,151]. Thus, in the past few years, they have been investigated as potential antitumor agents. In parallel, microtubules are tubulin polymers essential for cell growth, division and intracellular trafficking [28], and are known to be valuable targets for tumor therapy in women. Interestingly, several HDACis (CI-994, panabinstat, SAHA, SBHA, scriptaid and trichostatin A) and MTis (colchicine, nocodazole, paclitaxel and vinblastine) that have been tested in FMC cell lines show a dose-dependent antiproliferative effect and conserved cell death mechanism, by apoptosis. Furthermore, using HDACi it was possible to demonstrate an accumulation of the acetylated form of the histone H3 (Lys9/Lys14), as described in humans [9,152].

#### 4. Conclusions

The cat is considered a good oncology model [4], namely for HER2-positive and triple-negative breast cancers [17,55], although more efforts are needed to better understand the development mechanism and biology of FMC.

FMC tends to be diagnosed belatedly, presenting ulcerated masses, or metastasis [31], and the therapeutic alternatives available are scarce, being restricted to mastectomy [80] and adjuvant therapeutic protocols, with, however, limited success [4,31,50]. With this in mind, research groups are now directing their attention to the in vitro study of drugs already approved for HBC therapy on FMC cell-based models, demonstrating promising antiproliferative effects of several compounds. Furthermore, through the analysis of mammary carcinoma clinical samples, it has been possible to show that the cat does not present any known mutations thought to lead to resistance to therapy [10,11]. Moreover, similarities between the feline and human tumor micro- and serological environments have also been revealed, suggesting equivalent tumor diagnostic and prognostic biomarkers, as well as the possible use of adjuvant treatments recommended in breast cancer therapeutic protocols [64,66,153,154]. This introduces a new research line, e.g., the use of anti-leptin [153], anti-PD1 [154], or anti-VEGF [155] molecules.

Forthcoming perspectives include a deeper knowledge of FMC, defining proper diagnostic and prognostic biomarkers that can be used in clinical practice, and improvement of therapeutic options for cats. Additionally, we point out that, in the near future, a fast diagnostic kit to identify serum protein expression levels in cats with mammary carcinomas may become available, e.g., in the HER2-positive subtype, one of the most aggressive tumors [17], which would allow prediction of prognosis and inform the choice of therapeutic protocol.

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