



Canine invasive mammary carcinomas as models of human breast cancer. Part 1: natural history and prognostic factors

Frédérique Nguyen, Laura Pena, Catherine Ibisch, Delphine Loussouarn, Adelina Gama, Natascha Rieder, Anton Belousov, Mario Campone, Jérôme Abadie

► To cite this version:

Frédérique Nguyen, Laura Pena, Catherine Ibisch, Delphine Loussouarn, Adelina Gama, et al.. Canine invasive mammary carcinomas as models of human breast cancer. Part 1: natural history and prognostic factors. Breast Cancer Research and Treatment, 2017, Epub ahead of print. 10.1007/s10549-017-4548-2 . inserm-01629480

HAL Id: inserm-01629480


<https://inserm.hal.science/inserm-01629480>

Submitted on 6 Nov 2017

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Canine invasive mammary carcinomas as models of human breast cancer. Part 1: natural history and prognostic factors

Frédérique Nguyen^{1,2}  · Laura Peña³ · Catherine Ibisch^{1,2} · Delphine Loussouarn^{2,4} · Adelina Gama⁵ · Natascha Rieder⁶ · Anton Belousov⁷ · Mario Campone^{2,8} · Jérôme Abadie^{1,2}

Received: 13 October 2017 / Accepted: 16 October 2017
© The Author(s) 2017. This article is an open access publication

Abstract

Purpose Dogs have been proposed as spontaneous animal models of human breast cancer, based on clinicopathologic similarities between canine and human mammary carcinomas. We hypothesized that a better knowledge of the natural history and prognostic factors of canine invasive mammary carcinomas would favor the design of preclinical trials using dogs as models of breast cancer.

Methods The 2-year outcome of 350 female dogs with spontaneous invasive mammary carcinoma was studied. The investigated prognostic factors included age at diagnosis,

pathologic tumor size, pathologic nodal stage, lympho-vascular invasion, histological grade, and expression of Estrogen Receptor alpha (ER α), Progesterone Receptor, Ki-67, Human Epidermal Growth Factor Receptor 2, basal cytokeratins 5/6, and Epidermal Growth Factor Receptor. Multivariate survival analyses were performed using the Cox proportional hazards model.

Results The overall survival after mastectomy was 11 months. Within 1 year post mastectomy, 41.5% of dogs (145/350) died from their mammary carcinoma. By multivariate analysis, the significant prognostic factors for overall survival included a pathologic tumor size larger than 20 mm [HR 1.47 (95% confidence interval 1.15–1.89)], a positive nodal stage [pN+, HR 1.89 (1.43–2.48)], a histological grade III [HR 1.32 (1.02–1.69)], ER α negativity [HR 1.39 (1.01–1.89)], a high Ki-67 proliferation index [HR 1.32 (1.04–1.67)], and EGFR absence [HR 1.33 (1.04–1.69)].

Conclusion The short natural history of spontaneous canine invasive mammary carcinomas and high rate of cancer-related death allow for rapid termination of preclinical investigations. The prognostic factors of invasive mammary carcinomas are remarkably similar in dogs and humans, highlighting the similarities in cancer biology between both species.

Keywords Dog · Spontaneous animal model · Breast cancer · Estrogen Receptor alpha · HER2 · Prognosis

List of Abbreviations

95%-CI	95% confidence interval
CK5/6	Cytokeratins 5 and 6
CMC	Canine mammary carcinoma
DFI	Disease-free interval
DMFI	Distant metastasis-free interval
EGFR	Epidermal growth factor receptor (type 1)

✉ Frédérique Nguyen
frederique.nguyen@oniris-nantes.fr

¹ Oniris, Nantes Atlantic College of Veterinary Medicine Food Science and Engineering, Animal Cancers, Models for Research in Comparative Oncology (AMaROC) Research Unit, Site de La Chantrerie, 102 Route de Gachet, CS40706, 44307 Nantes, France

² CRCINA, INSERM, Université d'Angers, Université de Nantes, Nantes, France

³ Department of Animal Medicine, Surgery and Pathology, Complutense University of Madrid, Madrid, Spain

⁴ Department of Pathology, University Hospital, Nantes, France

⁵ Animal and Veterinary Research Centre (CECAV), University of Trás-os-Montes and Alto Douro (UTAD), Vila Real, Portugal

⁶ Pathology and Tissue Analytics, Pharma Research & Early Development, Roche Innovation Center Munich, Munich, Germany

⁷ Pharmaceutical Sciences, Pharma Research & Early Development, Roche Innovation Center Munich, Munich, Germany

⁸ Institut de Cancérologie de l'Ouest, Angers, France

ER α	Estrogen receptor alpha
HER2	Human epidermal growth factor receptor type 2
HES	Hematoxylin–eosin–safran
HR	Hazard ratio
IHC	Immunohistochemistry
LRR	Locoregional relapse
LVI	Lymphovascular invasion
M	Distant metastasis
OS	Overall survival
PR	Progesterone Receptor
pT	Pathologic tumor size
pN	Pathologic nodal stage
SS	Specific survival

Introduction

Breast cancer represents the most prevalent cancer and the leading cause of cancer death in women worldwide [1]. Despite considerable progress in breast cancer management, prognosis in the metastatic setting remains poor. The 5-year specific survival after initial diagnosis was estimated 97% for stage I, 88% for stage II, 70% for stage III, and only 25% for stage IV breast cancer [2]. One of the current challenges is to define molecular tools and relevant models that can predict the response and potential resistance to therapies. The classic *in vitro* (tumor cell lines) and *in vivo* (xenografts) preclinical models have indeed limitations related to the difficulty to reproduce interactions with the microenvironment, the absent or incomplete metastatic pattern, and their inability to fully integrate the host immune response [3]. Spontaneous tumor models are thus of high interest, to study the pharmacokinetics of innovative therapeutics *in vivo*, their effect on tumor (pathologic response) and patient (metastasis, survival), and the interactions between tumor cells and their microenvironment. In this respect, canine spontaneous cancers seem particularly relevant to human oncology [4–6].

Although their prevalence decreases in regions where early preventive ovariectomy is routinely performed, canine mammary carcinomas (CMCs) remain the most common canine cancer, with an estimated annual incidence of 182 per 100,000 female dogs [7]. Recent publications describe the relevance of spontaneous CMCs as models of human breast cancer, because of their high incidence, similarities in relative age of onset, risk factors, biological behavior, and metastatic pattern [8–11]. However, the biological behavior of CMCs needs further evaluation. Few studies dealt with the natural history of CMCs, *i.e.*, the outcome of dogs after mastectomy as single therapy [12–17]. The prognostic factors of CMCs were poorly described, usually in medium-sized cohorts (45–229 dogs), and mostly by univariate analyses [15–29], although multivariate analyses are available [14, 30–41]. Because adjuvant chemotherapy does

not significantly improve survival in dogs with advanced invasive CMC [41], and because tamoxifen-based hormone therapy is associated with significant adverse effects [42], most dogs benefit only from mastectomy, sometimes associated with ovariectomy [43]. This situation allows studies of the natural history of invasive CMCs, and the identification of prognostic factors without the confounding effects of adjuvant therapy. This also favors preclinical therapeutic trials of new anti-cancer drugs as first-line regimens, rather than in relapsed patients with advanced cancer.

Here, we hypothesized that (1) knowledge of the natural history of CMCs would emphasize the aggressive and short course of the disease, and could be useful for the design of preclinical therapeutic trials in dogs with CMC, as translational models of human breast cancer; (2) knowledge of the prognostic factors of CMCs would highlight the biological similarities between spontaneous CMCs and breast cancers.

The aims of this study were thus to describe the natural history of invasive CMCs, *i.e.*, cancer progression and mortality rates, in the largest cohort collected so far (350 female dogs); to describe invasive CMCs using human pathological criteria including immunohistochemical markers; and to validate these criteria as prognostic factors able to predict patients' outcome. In part 2 of this article, we evaluated the prognostic significance of the immunohistochemical classification of human breast cancer applied to dogs.

Methods

Patients and samples

This retrospective study included 350 female dogs with invasive mammary carcinoma, but free from other cancer, initially diagnosed in two laboratories of veterinary histopathology (Laboratoire d'Histopathologie Animale, Oniris, Nantes, and Laboratoire d'Anatomie Pathologique Vétérinaire d'Amboise, France) between 2007 and 2010. The owners' written consent and approval from the Oniris College of Veterinary Medicine local Animal Welfare Committee were obtained prior to inclusion.

Dogs were eligible for inclusion when a histological diagnosis of invasive mammary carcinoma was established and confirmed by an absent layer of p63-positive myoepithelial cells (anti-p63 antibody, clone ab111449, Abcam) by immunohistochemistry (IHC) that differentiates invasive from *in situ* mammary carcinomas [44, 45]. All dogs were treated surgically by their veterinarian, and none of them received any additional treatment before and/or after mastectomy. Age, breed, spay status, parity, contraception, prior benign mammary lesions, medical history, and outcome were obtained through written questionnaires or telephone interviews with referring veterinarians and owners. All 350

dogs were followed for at least 48 months with particular emphasis on the occurrence of locoregional relapse (time between mastectomy and the earliest local recurrence on the same mammary gland, new primary mammary tumor, or lymph node metastasis), distant metastasis-free interval (time from mastectomy to first evidence of distant metastases by medical imaging), and disease-free interval (interval from mastectomy to the first local recurrence, new primary tumor, lymph node metastasis, and/or distant metastasis). Overall survival was defined as the time between mastectomy and death from any cause. Specific survival was defined as the time between mastectomy and death attributable to the mammary carcinoma.

Pathological evaluation

Histological examination was performed on 3- μ m-thick hematoxylin–eosin–safran (HES) stained sections. The 350 tumors were classified according to the human breast cancer classification adapted to dogs (World Health Organization classification system) [46, 47], and graded according to the criteria of Elston and Ellis [48] adapted to canine mammary carcinomas [38]. The pathologic tumor size (pT, measured on histological slides), lymphovascular invasion (LVI), dermal infiltration, cutaneous ulceration, muscle invasion, margin status, and central necrosis were recorded for each case. Peritumoral lymphohistiocytic inflammation was considered positive when moderate to severe. In case of multicentric CMC, the largest tumor and/or tumor of highest histological grade was considered for prognostic purposes.

The methods used for IHC were detailed previously [35]. Briefly, automated IHC (Benchmark XT Ventana, Roche Diagnostics) was performed on 3- μ m-thick serial sections using the following antibodies: monoclonal mouse anti-human Estrogen Receptor alpha (ER α , clone C311, Santa Cruz, dilution 1:50), monoclonal rabbit anti-human Progesterone Receptor (PR, clone 1E2, Roche Diagnostics, prediluted), monoclonal rabbit anti-Human Epidermal Growth Factor Receptor Type 2 (HER2, clone 4B5, Roche Diagnostics, prediluted), polyclonal rabbit anti-HER2 (Dako A0485, dilution 1:400), monoclonal mouse anti-human Ki-67 (clone MIB1, Dako, dilution 1:50), monoclonal mouse anti-human Cytokeratins 5/6 (CK5/6, clone D5/16B4, Dako, dilution 1:50), and monoclonal mouse anti-Epidermal Growth Factor Receptor Type 1 (EGFR, clone 31G7, Invitrogen, dilution 1:20).

ER α , PR, and Ki-67 were assessed based on the number of positive nuclei among > 500 neoplastic cells (manual image analysis, Image J software, National Institute of Health, Bethesda, Maryland, USA), and considered positive at threshold $\geq 10\%$ for ER α and PR [45, 49], CK5/6, and EGFR [50]. The > 33.3% threshold for Ki-67 was evaluated by the receiver-operator-characteristic curve calculated for

2-year cancer-specific mortality. HER2 was scored 0 for no staining at all or incomplete, faint/barely perceptible membrane staining in $\leq 10\%$ of tumor cells; 1 + for incomplete and faint/barely perceptible membrane staining in > 10% of tumor cells; 2 + for circumferential and incomplete and/or weak/moderate membrane staining in > 10% of tumor cells; or incomplete and circumferential membrane staining that is intense but within $\leq 10\%$ of tumor cells; and 3 + for circumferential, complete, and intense membrane staining in > 10% of tumor cells. Carcinomas were considered HER2 positive only for a 3 + IHC score [45, 51].

Negative controls were included in each IHC run, and consisted in replacing the primary antibody with normal mouse or rabbit serum (prediluted reagents, Roche Diagnostics). The positive controls were mostly internal (epidermis and hair follicles for Ki-67, CK5/6, and EGFR; non-neoplastic mammary gland surrounding the carcinoma for ER α and PR; sebaceous glands for ER α). For HER2, the pathway HER2 4-in-1 control slides (Roche Diagnostics) were chosen to assess the quality of staining for each HER2 score (0, 1 +, 2 +, 3 +).

Four veterinary pathologists (JA, FN, LP, AG) and 1 medical pathologist (DL) examined the HES and IHC slides blindly (i.e., without any information on the dog or on the other pathologists' interpretation). In case of discrepancy between evaluators, cases were collectively reviewed in order to achieve a consensual diagnosis, grade, and immunohistochemical scoring.

Statistical analyses

The MedCalc[®] statistical software (Ostend, Belgium) was used. Continuous variables are expressed as median, [range], mean \pm standard deviation. Correlations between categorical variables were analyzed using the Pearson Chi-square test. The Kaplan–Meier method and log-rank tests were used for univariate survival analyses, and Cox proportional hazards models for multivariate survival analyses, whose results are reported using the Hazard Ratio (HR), its confidence interval (95%-CI), and the *p* value of each covariate. For all statistical tests, a *p* value < 0.05 was considered significant.

Results

Clinicopathologic features of invasive canine mammary carcinomas (CMCs)

The cohort comprises 350 female dogs with invasive CMC, including 253 (72.3%) intact and 97 (27.7%) spayed female dogs. The main characteristics of patients and CMCs are given in Table 1. The mean age at diagnosis was 11.0 ± 2.1 years [range (3.6–16.3), median 11.0 years].

Table 1 Characteristics of dogs and their invasive mammary carcinoma at diagnosis

	<i>N</i>	%
Age (years) (<i>n</i> = 349) ^a		
< 11.7 years	229	65.6
≥ 11.7 years	120	34.4
Spay status (<i>n</i> = 350)		
Intact females	253	72.3
Neutered females	97	27.7
Multicentricity (<i>n</i> = 350)		
Single carcinoma	295	84.3
Multicentric carcinoma	55	15.7
Pathologic tumor size (<i>n</i> = 350)		
pT < 20 mm	141	40.3
pT ≥ 20 mm	209	59.7
Pathologic nodal stage (<i>n</i> = 350)		
pN0	39	11.1
pNX	236	67.4
pN+	75	21.4
Lymphovascular invasion (<i>n</i> = 350)		
LVI+	171	48.9
LVI−	179	51.1
Histological grade (<i>n</i> = 350)		
I	19	5.4
II	106	30.3
III	225	64.3
Histological type (<i>n</i> = 350)		
Invasive mammary carcinoma	350	100
Simple tubulopapillary	176	50.3
Simple solid	103	29.4
Complex	31	8.9
Anaplastic	21	6.0
Squamous cell	14	4.0
Inflammatory	5	1.4
Surgical margins (<i>n</i> = 350)		
Positive margins	158	45.1
Negative margins	192	54.9
Peritumoral inflammation (<i>n</i> = 350)		
Yes (moderate to severe)	168	48.0
No (absent to mild)	182	52.0
ERα (<i>n</i> = 350)		
ER+ (≥ 10%)	57	16.3
ER− (< 10%)	293	83.7
PR (<i>n</i> = 350)		
PR+ (≥ 10%)	40	11.4
PR− (< 10%)	310	88.6
Ki-67 (<i>n</i> = 350)		
Ki-67 low (≤ 33.3%)	162	46.3
Ki-67 high (> 33.3%)	188	53.7
HER2 clone 4B5 (<i>n</i> = 350)		
0	246	70.3
1 +	76	21.7

Table 1 (continued)

	<i>N</i>	%
2 +	28	8.0
3 +	0	0
HER2 polyclonal Dako (<i>n</i> = 350)		
0	262	74.9
1 +	71	20.3
2 +	17	4.9
3 +	0	0
CK5/6 (<i>n</i> = 350)		
CK5/6+ (≥ 10%)	229	65.4
CK5/6− (< 10%)	121	34.6
EGFR (<i>n</i> = 350)		
EGFR+ (≥ 10%)	186	53.1
EGFR− (< 10%)	164	46.9

^aOne case with missing data

Fifty-seven breeds were represented. Mixed-breed dogs (*n* = 78, 22.3%) outnumbered Poodles (*n* = 50; 14.3%), German Shepherds (*n* = 25; 7.1%), Brittany and Labrador Retrievers (*n* = 19 each; 5.4%), and Yorkshire Terriers (*n* = 10; 2.9%).

In 235 dogs (67.1%), the invasive carcinoma was the first mammary lesion detected, whereas 115 (32.9%) dogs had a history of previous non-malignant mammary lesions. Parity was unknown in 269 dogs (76.9%), and nulliparous females (*n* = 49; 14.0%) slightly outnumbered multiparous (*n* = 32; 9.1%) females. History of contraceptive use was reported in 20 (5.7%) dogs.

Tumors involved the abdominal and inguinal mammary glands (M3 to M5) in 256 cases (73.1%), the thoracic mammary glands (M1–M2) in 50 (14.3%), both in 11 (3.1%), and location was unrecorded in 33 cases (9.4%). The most common surgical procedure was radical mastectomy (excision of the 5 mammary glands of the affected side) in 156 dogs (44.6%), followed by regional (M1–M3 or M3–M5) mastectomy in 112 cases (32.0%), and single mastectomy in 64 cases (18.3%). Information on the surgical procedure was missing in 18 dogs (5.1%).

The mean pathologic tumor size was 18 ± 7 mm [median 18 mm, range (2–49), *n* = 227 dogs]; in the other cases, the pathologic tumor size could not be precisely determined due to larger size and/or positive margins. In 236 dogs (67.4%), the pathologic nodal stage was pNX due to absence of lymph node sampling for histopathology. Nodal stage pN+ (with metastasis of any size) was confirmed in 75 cases (21.4%). Six dogs (1.7%) had evidence of distant metastasis (M1) at diagnosis.

All of the included cases correspond to invasive mammary carcinomas according to breast cancer classification. The predominant histological types were simple

tubulopapillary ($n = 176$; 50.3%), simple solid ($n = 103$; 29.4%), and complex carcinomas (malignant epithelial proliferation associated with benign myoepithelial proliferation, $n = 31$, 8.6%). The mean mitotic index was 41 ± 29 mitoses in 10 high-power fields [$\times 400$, diameter of the field of view 0.55 mm; median 33, range (5–236)].

Regarding histopathological criteria of aggressiveness, dermal infiltration was present in 119 cases (34.0%), cutaneous ulceration in 50 cases (14.3%), abdominal or thoracic muscle infiltration in 65 cases (18.6%), peritumoral inflammation in 168 cases (48.0%), and central necrosis in 261 cases (74.6%).

The mean ER α index was $6.3 \pm 14.0\%$ (0–87.6%); 58.0% of cases ($n = 203$) did not express ER α at all. The mean PR index was $5.4 \pm 14.8\%$ (0–92.0%); 65.4% ($n = 229$) of CMCs did not express PR at all. At positive threshold 10%, 57 CMCs (16.3%) were ER + and 40 (11.4%) were PR+ (Fig. 1). Two hundred and sixty-seven CMCs (76.3%) were ER – PR –, 26 (7.4%) were ER – PR +, 43 (12.3%) were ER + PR –, and only 14 (4.0%) were ER + PR +. The

mean Ki-67 index was $36.2 \pm 17.4\%$ [median 35.4%, range (1.3–94.6%)].

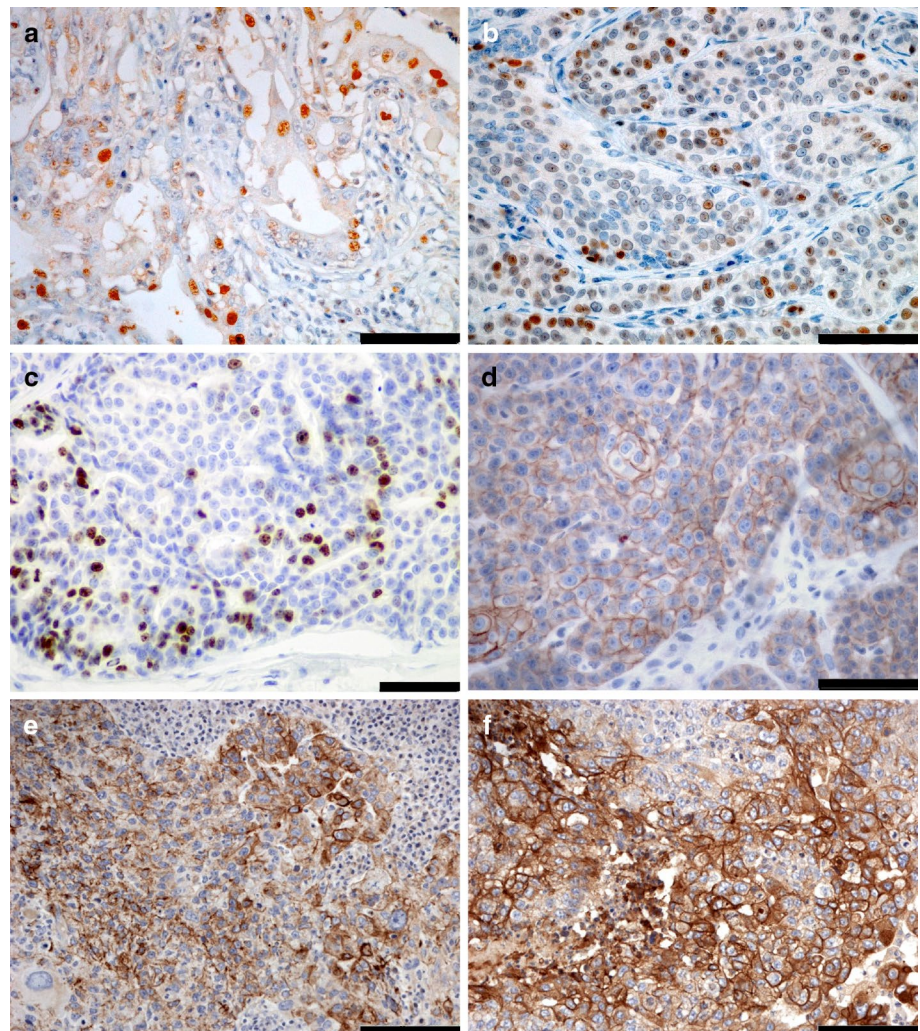
Both immunohistochemical protocols used to assess HER2 expression were highly correlated ($p < 0.0001$, Chi-square test). HER2 score 0 was predominant (70.3% of the cases with clone 4B5, 74.9% with polyclonal A0485), followed by HER2 score 1 + (Table 1). The cohort does not comprise any case with HER2 overexpression (score 3 +).

Natural history and prognostic factors of canine invasive mammary carcinoma

Locoregional relapse (LRR)

The median time to LRR was 26.4 months; the LRR probability was 34% at 1 year, and 47% at 2 years post diagnosis (Fig. 2a). At the end of the follow-up period, 76 dogs (21.7%) had experienced tumor recurrence at the site of prior mastectomy, 56 dogs (16.0%) a new primary mammary tumor, and 18 dogs (5.1%) more than one locoregional event.

Fig. 1 Immunohistochemical markers of canine invasive mammary carcinomas. Positivity to **a** Estrogen Receptor alpha (ER α , nuclear), **b** Progesterone Receptor (PR, nuclear), **c** the proliferation index Ki-67 (nuclear), **d** score 2 + for Human Epidermal Growth Factor Receptor type 2 (HER2, membranous), and positivity to **e** basal cytokeratins 5 and 6 (CK5/6, cytoplasmic), and **f** Epidermal Growth Factor Receptor type 1 (EGFR, membranous) in 6 different canine invasive mammary carcinomas. Indirect immunohistochemistry, initial magnification $\times 400$, bar = 50 micrometers



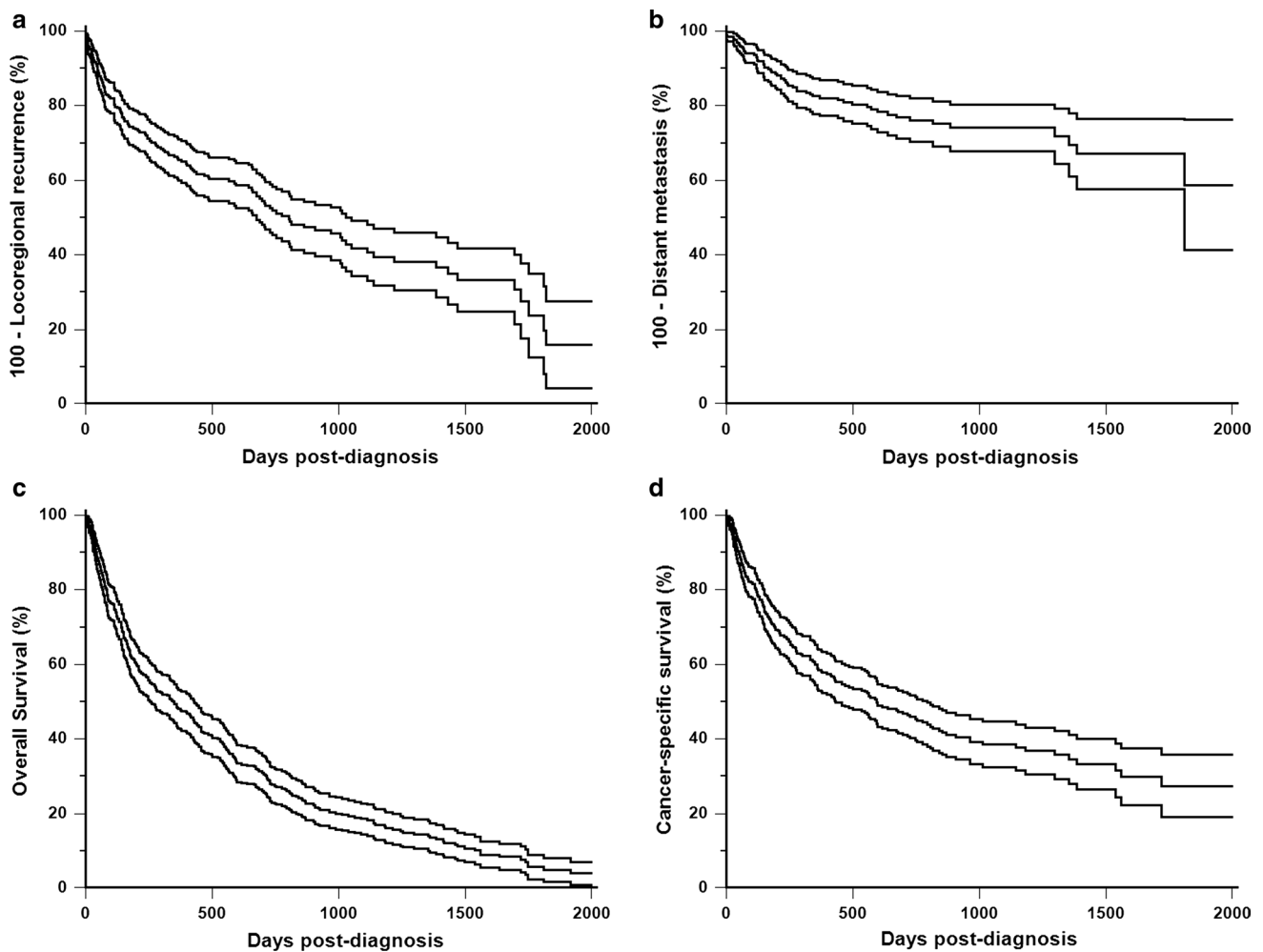


Fig. 2 Natural history of invasive mammary carcinoma in 350 female dogs. Kaplan–Meier curves for **a** Locoregional Relapse (LRR), **b** Distant Metastasis-Free Interval (DMFI), **c** Overall Survival

(OS), and **d** Specific Survival (SS). The 95% confidence interval is shown for each survival curve

By univariate analysis, 11 parameters were significantly associated with the LRR risk (Table 2), of which 4 remained as significant independent prognostic factors by multivariate analysis ($p < 0.0001$): the strongest predictor of locoregional relapse was ER α positivity (HR 0.48), followed by the pathological nodal stage pN + (HR 1.92), the presence of lymphovascular invasion, and positive margins (HR = 1.55 for each).

Distant Metastasis-Free Interval (DMFI)

The risk of distant metastasis was 17% at 1 year and 24% at 2 years post diagnosis (Fig. 2b), and was likely underestimated in this retrospective cohort, as the dogs' owners may have declined complete staging, for financial reasons.

By univariate analysis, six parameters were significantly associated with DMFI (Table 3), of which four remained as significant independent prognostic factors by multivariate

analysis ($p < 0.0001$): the strongest was lymphovascular invasion (HR 2.66), followed by age at diagnosis (HR 2.16 for older dogs), multicentricity (HR 1.89), and the Ki-67 proliferation index (HR 1.0149).

Disease-free interval (DFI)

The median DFI was 34.4 months. Cancer progression (locoregional recurrence and/or distant metastasis) was recorded in 34% of dogs at 1 year post diagnosis, and 45% at 2 years.

By univariate analysis, 13 parameters were significantly associated with DFI (Table 4), of which 4 were independent prognostic factors by multivariate analysis ($p < 0.0001$): the pathologic nodal stage pN + (HR 1.92), ER α negativity (HR 1.69), a high proliferation index (HR 1.59), and positive margins (HR 1.54).

Table 2 Prognostic factors for Locoregional Relapse of canine invasive mammary carcinomas by univariate and multivariate analyses

Univariate analysis	HR	95%-CI	<i>p</i>
Breed			
Molossoid breeds	2.26	1.10–4.64	0.0266
British and Irish pointing dogs	4.61	1.74–12.20	0.0022
Japanese, Chinese and Pekingese Spaniels	8.22	1.11–61.09	0.0406
Continental Toy Spaniels	11.56	1.52–87.73	0.0185
Molossian Toy dogs	16.40	2.17–123.95	0.0070
Any other breed	1.00	Reference	
Histological type			
Anaplastic CMC	2.38	1.19–4.77	0.0148
Inflammatory CMC	12.22	4.30–34.74	< 0.0001
Any other type	1.00	Reference	
Lymphovascular invasion			
LVI– versus LVI+	0.49	0.35–0.69	< 0.0001
Pathologic nodal stage			
pN+ versus pN0–pNX	2.31	1.46–3.67	< 0.0001
Margin status			
Positive versus negative margins	1.86	1.33–2.61	0.0001
Central necrosis			
Absent versus present	1.46	0.99–2.18	0.0343
Peritumoral inflammation			
No versus yes	0.67	0.48–0.92	0.0105
ERα			
ER+ versus ER–	0.49	0.33–0.73	0.0036
PR			
PR+ versus PR–	0.54	0.34–0.88	0.0460
Ki-67			
Continuous (%)	1.0107	1.0017–1.0197	0.0227
EGFR			
EGFR+ versus EGFR–	0.72	0.52–0.99	0.0428
Multivariate analysis			
Lymphovascular invasion			
LVI+ versus LVI–	1.55	1.08–2.24	0.0181
Pathologic nodal stage			
pN+ versus pN0–pNX	1.92	1.30–2.84	0.0012
Margin status			
Positive versus negative margins	1.55	1.10–2.18	0.0135
ERα			
ER+ versus ER–	0.48	0.29–0.79	0.0040

Overall survival (OS)

During the follow-up period, 310 dogs (88.6%) died (Fig. 2c). The median OS was 11.4 months (2 days–75 months). The mortality rate was 51.7% at 1 year and 72.0% at 2 years post diagnosis. Death was unrelated to cancer in 58 dogs (16.6%), from unknown causes in 65 dogs (18.6%), and attributable to the invasive CMC in 187 dogs (53.4%).

By univariate analysis, 16 parameters were significantly associated with OS (Table 5), of which 6 were independent prognostic factors by multivariate analysis ($p < 0.0001$). The strongest prognostic factors were the pathologic nodal stage (pN + : HR 1.89) and pathologic tumor size (pT \geq 20 mm: HR 1.47), followed by the histological grade, ER α positivity, the Ki-67 index, and EGFR expression (HR 1.32–1.39).

Table 3 Prognostic factors for Distant Metastasis-Free Interval (DMFI) of dogs with invasive mammary carcinomas ($n = 350$)

Univariate analysis	HR	95%-CI	<i>p</i>
Age at diagnosis			
≤ 11.7 versus > 11.7 years	0.44	0.25–0.75	0.0007
Multicentricity			
Single versus multicentric	0.44	0.20–0.96	0.0047
Lymphovascular invasion			
LVI– versus LVI+	0.33	0.20–0.56	<0.0001
Pathologic nodal stage			
pN+ versus pN0–pNX	1.86	0.92–3.75	0.0326
Peritumoral inflammation			
No versus yes	0.58	0.35–0.96	0.0281
Ki-67			
Continuous (%)	1.0203	1.0068–1.0339	0.0045
Multivariate analysis			
Age at diagnosis			
> 11.7 versus ≤ 11.7 years	2.16	1.29–3.62	0.0037
Multicentricity			
Multicentric versus single	1.89	1.03–3.46	0.0404
Lymphovascular invasion			
LVI+ versus LVI–	2.66	1.56–4.53	0.0003
Ki-67			
Continuous (%)	1.0149	1.0007–1.0293	0.0412

Specific survival

The median time to death attributable to cancer was 19.5 months [2 days–56 months] (Fig. 2d). The cancer-related death rate was 41.5% at 1 year and 54.1% at 2 years post diagnosis.

By univariate analysis, 15 clinicopathologic parameters were significantly associated with cancer-related death (Table 6), of which six were independent prognostic factors by multivariate analysis ($p < 0.0001$). The most significant predictors of cancer-related death were those that define the stage of invasive CMCs: the pathologic tumor size ($pT \geq 20$ mm: HR 1.41), pathologic nodal stage ($pN +$: HR = 1.82), and the presence of distant metastases at diagnosis (M1: HR 2.61). Peritumoral inflammation (HR 1.54), ER α negativity (HR 1.56), and a high Ki-67 proliferation index (HR 1.67) were also associated with cancer-related death, independently of the stage of the carcinoma at diagnosis.

Discussion

Dogs with invasive mammary carcinomas have been proposed as a useful resource for preclinical research in comparative oncology due to epidemioclinical, biological, and

pathological similarities with human breast cancer [8–11]. There was, however, a relative uncertainty of predictability of this spontaneous cancer as a translational model, as the natural history and prognostic factors have been described in relatively small cohorts [19–25, 27–41]. The present study is of particular interest because (1) mammary carcinomas *in situ* have been carefully excluded from analysis, using p63 immunohistochemistry when necessary, which is rarely, if ever, performed in veterinary studies, but of paramount importance in human breast oncology; (2) the cases were reviewed blindly by veterinary and medical pathologists, until consensus diagnoses were achieved, which permitted interpretation of canine samples using the criteria used for human breast cancer; (3) this is the largest cohort of CMCs described so far, which allowed for multivariate survival analyses with sufficient statistical power; (4) this study is one of the rare reports [14, 15, 17, 30, 37] of locoregional recurrence, distant metastasis-free interval, and specific survival in dogs with CMCs, as most previous studies focused on disease-free survival and overall survival only [18–21, 23–25, 28, 29, 31, 32, 35, 38, 39].

The epidemiological characteristics of CMCs in this cohort, although in agreement with some previous reports [20, 33], are characterized by an older age at diagnosis, lower rate of positivity to ER α and PR, and higher Ki-67 index than previous descriptions [19, 23, 27, 31, 37, 39].

Table 4 Prognostic factors for Disease-Free Interval of dogs with invasive mammary carcinomas ($n = 350$)

Univariate analysis	HR	95%-CI	<i>p</i>
Age at diagnosis			
Continuous (years)	1.1511	1.0624–1.2473	0.0006
Breed			
British and Irish pointing dogs	3.60	1.38–9.43	0.0094
Continental Toy Spaniels	22.64	5.10–100.46	< 0.0001
Molossian Toy dogs	15.41	2.04–116.71	0.0084
Any other breed	1.00	Reference	
Multicentricity			
Single versus multicentric	0.50	0.30–0.84	0.0007
Histological type			
Anaplastic CMC	2.11	1.01–4.38	0.0468
Inflammatory CMC	6.81	1.63–28.54	0.0090
Any other type	1.00	Reference	
Histological grade			
I versus II–III	0.36	0.15–0.89	0.0284
Lymphovascular invasion			
LVI– versus LVI+	0.35	0.25–0.49	< 0.0001
Pathologic nodal stage			
pN+ versus pN0–pNX	2.20	1.37–3.51	< 0.0001
Muscle invasion			
No versus yes	0.67	0.42–1.06	0.0482
Margin status			
Positive versus negative margins	1.63	1.15–2.30	0.0031
Peritumoral inflammation			
No versus yes	0.65	0.46–0.91	0.0093
ER α			
ER+ versus ER–	0.57	0.38–0.85	0.0222
Ki-67			
Continuous (%)	1.0164	1.0073–1.0256	0.0006
EGFR			
EGFR+ versus EGFR–	0.72	0.51–1.00	0.0473
Multivariate analysis			
Margin status			
Positive versus negative margins	1.54	1.09–2.16	0.0140
Pathologic nodal stage			
pN+ versus pN0–pNX	1.92	1.30–2.82	0.0011
ER α			
ER + versus ER–	0.59	0.36–0.97	0.0367
Ki-67			
$\leq 33.3\%$ versus $> 33.3\%$	0.63	0.45–0.89	0.0096

These differences can be attributed at least in part to the systematic exclusion of mammary carcinomas in situ, which are diagnosed in younger dogs, and are more commonly ER α and PR positive compared to invasive CMCs (unpublished observations, manuscript in preparation), as described in human breast cancer [52].

Another particularity of this cohort is the absence of any HER2-positive CMC, as previously reported [53]. However, HER2-positive CMCs have been previously described by immunohistochemistry, with the polyclonal A0485 antibody [18, 26, 54–58] or the CB11 clone [59]. In this study, the external positive controls (cytospins of breast carcinoma cell

Table 5 Prognostic factors for Overall Survival of dogs with invasive mammary carcinomas ($n = 350$)

Univariate analysis	HR	95%-CI	<i>p</i>
Age at diagnosis			
Continuous (years)	1.1508	1.0898–1.2152	< 0.0001
History of contraception			
No versus yes/unknown	0.73	0.57–0.93	0.0106
Multicentricity			
Single versus multicentric	0.57	0.40–0.82	0.0001
Histological type			
Anaplastic CMC	3.45	2.18–5.46	< 0.0001
Inflammatory CMC	11.56	4.66–28.67	< 0.0001
Any other type	1.00	reference	
Histological grade			
I versus III	0.36	0.20–0.64	0.0006
II versus III	0.71	0.55–0.91	0.0066
Pathologic tumor size			
< 1 cm versus ≥ 2 cm	0.49	0.30–0.81	0.0054
1 cm \leq pT < 2 cm versus ≥ 2 cm	0.65	0.51–0.83	0.0006
Lymphovascular invasion			
LVI– versus LVI+	0.42	0.34–0.54	< 0.0001
Pathologic nodal stage			
pN0 versus pNX	0.60	0.41–0.88	0.0091
pN+ versus pNX	1.80	1.38–2.36	< 0.0001
Dermal invasion			
No versus yes	0.77	0.60–0.98	0.0276
Muscle invasion			
No versus yes	0.66	0.48–0.91	0.0029
Margin status			
Positive versus negative margins	1.99	1.57–2.52	< 0.0001
Peritumoral inflammation			
No versus yes	0.68	0.54–0.85	0.0005
ER α			
ER+ versus ER–	0.69	0.53–0.91	0.0169
Ki-67			
$\leq 33.3\%$ versus > 33.3%	0.65	0.52–0.81	0.0001
CK5/6			
CK5/6+ versus CK5/6–	0.78	0.62–0.99	0.0376
EGFR			
EGFR > 0 versus EGFR absent	0.76	0.58–0.98	0.0217
Multivariate analysis			
Pathologic tumor size			
pT < 20 mm versus pT ≥ 20 mm	0.68	0.53–0.87	0.0026
Pathologic nodal stage			
pN+ versus pN0–pNX	1.89	1.43–2.48	< 0.0001
Histological grade			
I–II versus III	0.76	0.59–0.98	0.0328
ER α			
ER+ versus ER–	0.72	0.53–0.99	0.0436
Ki-67			
$\leq 33.3\%$ versus > 33.3%	0.76	0.60–0.96	0.0228
EGFR			
EGFR absent versus EGFR > 0	1.33	1.04–1.69	0.0239

Table 6 Prognostic factors for Cancer-Specific Survival of dogs with invasive mammary carcinomas ($n = 350$)

Univariate analysis	HR	95%-CI	<i>p</i>
Age at diagnosis			
Continuous (years)	1.1423	1.0652–1.2249	0.0002
Breed group			
Mixed-breed	1.98	1.09–3.62	0.0264
British and Irish pointing dogs	3.40	1.42–8.17	0.0065
Continental Toy Spaniels	24.39	5.62–105.81	< 0.0001
Any other breed	1.00	Reference	
Distant metastasis			
M1 versus M0–MX	3.19	0.77–13.18	0.0031
Multicentricity			
Single versus multicentric	0.50	0.32–0.78	0.0001
Histological type			
Anaplastic CMC	3.29	1.87–5.78	< 0.0001
Inflammatory CMC	14.35	5.71–36.07	< 0.0001
Any other type	1.00	Reference	
Histological grade			
I versus III	0.41	0.20–0.84	0.0151
II versus III	0.63	0.45–0.87	0.0054
Pathologic tumor size			
< 1 cm versus ≥ 2 cm	0.46	0.23–0.90	0.0251
1 cm \leq pT < 2 cm versus ≥ 2 cm	0.70	0.51–0.95	0.0242
Lymphovascular invasion			
LVI– versus LVI+	0.31	0.23–0.42	< 0.0001
Pathologic nodal stage			
pN0 versus pNX	0.53	0.30–0.92	0.0242
pN + versus pNX	2.13	1.54–2.94	< 0.0001
Central necrosis			
No versus Yes	1.38	0.98–1.96	0.0444
Dermal invasion			
No versus Yes	0.74	0.54–1.00	0.0427
Margin status			
Positive versus negative margins	1.93	1.43–2.60	< 0.0001
Peritumoral inflammation			
No versus yes	0.59	0.44–0.79	0.0003
ER α			
ER + versus ER–	0.61	0.43–0.88	0.0209
Ki-67			
Continuous (%)	1.0179	1.0102–1.0257	< 0.0001
Multivariate analysis			
Pathologic tumor size			
pT < 20 mm versus pT ≥ 20 mm	0.71	0.52–0.95	0.0232
Pathologic nodal stage			
pN+ versus pN0–pNX	1.82	1.30–2.54	0.0005
Distant metastasis			
M1 versus M0–MX	2.61	1.14–5.99	0.0245
Peritumoral inflammation			
Yes versus no	1.54	1.14–2.07	0.0050
ER α			
ER+ versus ER–	0.64	0.41–0.97	0.0380

Table 6 (continued)

Multivariate analysis

Ki-67

≤ 33.3% versus > 33.3%	0.60	0.44–0.81	0.0011
------------------------	------	-----------	--------

lines, representative of the HER2 scores 0–3 +) ensured that HER2 expression was neither underestimated nor overestimated on the canine slides, a precaution that was rarely taken in veterinary oncology [26, 54]. Of note, HER2 gene amplification was not previously found in CMCs [60], and thus the existence of HER2-positive mammary carcinomas in dogs is still uncertain [61, 62].

The natural history of invasive CMCs is much shorter in dogs (54% cancer-related death at 2 years post diagnosis in this study) than in human breast cancer [2], probably in relation to shorter life expectancy in dogs, and the lack of adjuvant therapy, a situation that favors the setting of pre-clinical trials in the canine species. The effects of a given compound on patient survival, including in first-line regimen, is expected to be evaluable in short delays in dogs with CMCs, an advantage already highlighted for other canine cancers [4, 5]. The prognostic factors of invasive CMCs, described here in the largest retrospective cohort described so far, include the pathologic tumor size, pathologic nodal stage, lymphovascular invasion, histological grade, and ERα positivity, which are all also strong prognostic factors in human breast cancer [63], confirming the similar biology of invasive mammary carcinomas in both species.

Conclusions

The results of the present study confirm that canine invasive mammary carcinomas have a short disease course, which is predictable with clinicopathologic criteria close to those of human oncology. In the second part of this article, we hypothesized that CMCs could be subdivided into luminal and triple-negative cases with different outcomes, as in human breast cancer.

Acknowledgements The authors deeply acknowledge Dr. Chand Khanna (EthosDiscovery, EthosVeterinary Health) for his help in reviewing the manuscript. The authors thank Dr. Claire Hanzenne, Dr. Ingrid Bemelmans, Dr. Floriane Morio, and Dr. Clotilde de Brito, who helped in collecting the clinical and follow-up data. The authors also thank Dr. Laëtitia Jaillardon for her involvement in data analysis, the veterinary pathologists (Dr. Jean-Loïc Le Net, Dr. Virginie Théau, Dr. Pierre Lagourette, Dr. Olivier Albaric and Dr. Sophie Labrut) who performed the initial diagnoses of canine mammary carcinomas, as well as the technicians in histopathology (Mr. Bernard Fernandez, Mrs. Florence Lezin, and Mrs. Catherine Guéreaud) who made the slides. Finally, we thank the referring veterinarians and the owners of the dogs included in this study, who gave us the clinical and follow-up data.

Funding This work was supported by the French National Cancer Institute (INCa, Institut National du Cancer) with a grant for translational research (INCa-DHOS 2010, Pr M. Campone) and one grant for PhD students on translational research (Grant N°201108; 2011); and by Roche Diagnostics GmbH, Germany, which provided financial and technical support for the immunohistochemical characterization of the carcinomas.

Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The owners' written consent and approval from the Oniris College of Veterinary Medicine local Animal Welfare Committee were obtained prior to inclusion of each canine mammary carcinoma in this retrospective observational study.

Open Access This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (<http://creativecommons.org/licenses/by/4.0/>), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.

References

1. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M et al (2015) Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer* 136:E359–E386
2. Macià F, Porta M, Murta-Nascimento C, Servitja S, Guxens M, Burón A et al (2012) Factors affecting 5- and 10-year survival of women with breast cancer: an analysis based on a public general hospital in Barcelona. *Cancer Epidemiol* 36:554–559
3. Vargo-Gogola T, Rosen JM (2007) Modelling breast cancer: one size does not fit all. *Nat Rev Cancer* 7:659–672
4. Gordon I, Paoloni M, Mazcko C, Khanna C (2009) The Comparative Oncology Trials Consortium: using spontaneously occurring cancers in dogs to inform the cancer drug development pathway. *PLoS Med* 6:e1000161
5. Paoloni M, Khanna C (2008) Translation of new cancer treatments from pet dogs to humans. *Nat Rev Cancer* 8:147–156
6. Ranieri G, Gadaleta CD, Patruno R, Zizzo N, Daidone MG, Hansson MG et al (2013) A model of study for human cancer: spontaneous occurring tumors in dogs. Biological features and translation for new anticancer therapies. *Crit Rev Oncol Hematol* 88:187–197
7. Merlo DF, Rossi L, Pellegrino C, Ceppi M, Cardellino U, Capurro C et al (2008) Cancer incidence in pet dogs: findings of the Animal Tumor Registry of Genoa, Italy. *J Vet Intern Med* 22:976–984

8. Pinho SS, Carvalho S, Cabral J, Reis CA, Gärtner F (2012) Canine tumors: a spontaneous animal model of human carcinogenesis. *Transl Res* 159:165–172
9. Rivera P, von Euler H (2011) Molecular biological aspects on canine and human mammary tumors. *Vet Pathol* 48:132–146
10. Uva P, Aurisicchio L, Watters J, Loboda A, Kulkarni A, Castle J et al (2009) Comparative expression pathway analysis of human and canine mammary tumors. *BMC Genomics* 10:135
11. Vail DM, MacEwen EG (2000) Spontaneously occurring tumors of companion animals as models for human cancer. *Cancer Invest* 18:781–792
12. Benjamin SA, Lee AC, Saunders WJ (1999) Classification and behavior of canine mammary epithelial neoplasms based on life-span observations in beagles. *Vet Pathol* 36:423–436
13. Moulton JE, Rosenblatt LS, Goldman M (1986) Mammary tumors in a colony of beagle dogs. *Vet Pathol* 23:741–749
14. Rasotto R, Berlato D, Goldschmidt MH, Zappulli V (2017) Prognostic significance of canine Mammary tumor histologic subtypes: an observational cohort study of 229 cases. *Vet Pathol* 54:571–578
15. Santos AA, Lopes CC, Marques RM, Amorim IF, Gärtner MF, de Matos AJ (2012) Matrix metalloproteinase-9 expression in mammary gland tumors in dogs and its relationship with prognostic factors and patient outcome. *Am J Vet Res* 73:689–697
16. Schneider R, Dorn CR, Taylor DO (1969) Factors influencing canine mammary cancer development and postsurgical survival. *J Natl Cancer Inst* 43:1249–1261
17. Stratmann N, Failing K, Richter A, Wehrend A (2008) Mammary tumor recurrence in bitches after regional mastectomy. *Vet Surg* 37:82–86
18. Araújo MR, Campos LC, Damasceno KA, Gamba CO, Ferreira E, Cassali GD (2016) HER-2, EGFR, Cox-2 and Ki67 expression in lymph node metastasis of canine mammary carcinomas: association with clinical-pathological parameters and overall survival. *Res Vet Sci* 106:121–130
19. Chang CC, Tsai MH, Liao JW, Chan JP, Wong ML, Chang SC (2009) Evaluation of hormone receptor expression for use in predicting survival of female dogs with malignant mammary gland tumors. *J Am Vet Med Assoc* 235:391–396
20. Chang SC, Chang CC, Chang TJ, Wong ML (2005) Prognostic factors associated with survival two years after surgery in dogs with malignant mammary tumors: 79 cases (1998–2002). *J Am Vet Med Assoc* 227:1625–1629
21. Ferreira E, Bertagnolli AC, Cavalcanti MF, Schmitt FC, Cassali GD (2009) The relationship between tumour size and expression of prognostic markers in benign and malignant canine mammary tumours. *Vet Comp Oncol* 7:230–235
22. Gama A, Alves A, Schmitt F (2010) Expression and prognostic significance of CK19 in canine malignant mammary tumours. *Vet J* 184:45–51
23. Gama A, Alves A, Schmitt F (2008) Identification of molecular phenotypes in canine mammary carcinomas with clinical implications: application of the human classification. *Virchows Arch* 453:123–132
24. Karayannopoulou M, Kaldrymidou E, Constantinidis TC, Desiris A (2005) Histological grading and prognosis in dogs with mammary carcinomas: application of a human grading method. *J Comp Pathol* 133:246–252
25. Pérez Alenza MD, Peña L, Nieto AI, Castaño M (1997) Clinical and pathological prognostic factors in canine mammary tumors. *Ann Ist Super Sanita* 33:581–585
26. Ressel L, Puleio R, Loria GR, Vannozzi I, Millanta F, Caracappa S et al (2013) HER-2 expression in canine morphologically normal, hyperplastic and neoplastic mammary tissues and its correlation with the clinical outcome. *Res Vet Sci* 94:299–305
27. Sassi F, Benazzi C, Castellani G, Sarli G (2010) Molecular-based tumour subtypes of canine mammary carcinomas assessed by immunohistochemistry. *BMC Vet Res* 6:5
28. Szczubiał M, Łopuszynski W (2011) Prognostic value of regional lymph node status in canine mammary carcinomas. *Vet Comp Oncol* 9:296–303
29. Yamagami T, Kobayashi T, Takahashi K, Sugiyama M (1996) Prognosis for canine malignant mammary tumors based on TNM and histologic classification. *J Vet Med Sci* 58:1079–1083
30. Betz D, Schoenrock D, Mischke R, Baumgärtner W, Nolte I (2012) Postoperative treatment outcome in canine mammary tumors. Multivariate analysis of the prognostic value of pre- and postoperatively available information. *Tierarztl Prax Ausg K Kleintiere Heimtiere* 40:235–242
31. De Las Mulas JM, Millán Y, Dios R (2005) A prospective analysis of immunohistochemically determined estrogen receptor alpha and progesterone receptor expression and host and tumor factors as predictors of disease-free period in mammary tumors of the dog. *Vet Pathol* 42:200–212
32. Diessler ME, Castellano MC, Portiansky EL, Burns S, Idiart JR (2017) Canine mammary carcinomas: influence of histological grade, vascular invasion, proliferation, microvessel density and VEGFR2 expression on lymph node status and survival time. *Vet Comp Oncol* 15:450–461
33. Hellmén E, Bergström R, Holmberg L, Spångberg IB, Hansson K, Lindgren A (1993) Prognostic factors in canine mammary tumors: a multivariate study of 202 consecutive cases. *Vet Pathol* 30:20–27
34. Itoh T, Uchida K, Ishikawa K, Kushima K, Kushima E, Tamada H et al (2005) Clinicopathological survey of 101 canine mammary gland tumors: differences between small-breed dogs and others. *J Vet Med Sci* 67:345–347
35. Jaillardon L, Abadie J, Godard T, Campone M, Loussouarn D, Siliart B et al (2015) The dog as a naturally-occurring model for insulin-like growth factor type 1 receptor-overexpressing breast cancer: an observational cohort study. *BMC Cancer* 15:664
36. Misdorp W, Hart AA (1976) Prognostic factors in canine mammary cancer. *J Natl Cancer Inst* 56:779–786
37. Nieto A, Peña L, Pérez-Alenza MD, Sánchez MA, Flores JM, Castaño M (2000) Immunohistologic detection of estrogen receptor alpha in canine mammary tumors: clinical and pathologic associations and prognostic significance. *Vet Pathol* 37:239–247
38. Peña L, De Andrés PJ, Clemente M, Cuesta P, Pérez-Alenza MD (2013) Prognostic value of histological grading in noninflammatory canine mammary carcinomas in a prospective study with two-year follow-up: relationship with clinical and histological characteristics. *Vet Pathol* 50:94–105
39. Peña LL, Nieto AI, Pérez-Alenza D, Cuesta P, Castaño M (1998) Immunohistochemical detection of Ki-67 and PCNA in canine mammary tumors: relationship to clinical and pathologic variables. *J Vet Diagn Invest* 10:237–246
40. Philibert JC, Snyder PW, Glickman N, Glickman LT, Knapp DW, Waters DJ (2003) Influence of host factors on survival in dogs with malignant mammary gland tumors. *J Vet Intern Med* 17:102–106
41. Tran CM, Moore AS, Frimberger AE (2016) Surgical treatment of mammary carcinomas in dogs with or without postoperative chemotherapy. *Vet Comp Oncol* 14:252–262
42. Tavares WL, Lavalle GE, Figueiredo MS, Souza AG, Bertagnolli AC, Viana FA et al (2010) Evaluation of adverse effects in tamoxifen exposed healthy female dogs. *Acta Vet Scand* 52:67
43. Kristiansen VM, Peña L, Díez Córdova L, Illera JC, Skjerve E, Breen AM et al (2016) Effect of ovariectomy at the time of tumor removal in dogs with mammary carcinomas: a randomized controlled trial. *J Vet Intern Med* 30:230–241

44. Shamloula MM, El-Shorbagy SH, Saied EM (2007) P63 and cytokeratin8/18 expression in breast, atypical ductal hyperplasia, ductal carcinoma in situ and invasive duct carcinoma. *J Egypt Natl Canc Inst* 19:202–210
45. Peña L, Gama A, Goldschmidt MH, Abadie J, Benazzi C, Castagnaro M et al (2014) Canine mammary tumors: a review and consensus of standard guidelines on epithelial and myoepithelial phenotype markers, HER2, and hormone receptor assessment using immunohistochemistry. *Vet Pathol* 51:127–145
46. Goldschmidt M, Peña L, Rasotto R, Zappulli V (2011) Classification and grading of canine mammary tumors. *Vet Pathol* 48:117–131
47. Misdorp W, Else RW, Hellmen E, Lipscomb TP (1999) Histological classification of mammary tumors of the dog and cat. 2nd series. Armed Forces Institute of Pathology, Washington, DC
48. Elston CW, Ellis IO (1991) Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. *Histopathology* 19:403–410
49. Gama A, Gärtner F, Alves A, Schmitt F (2009) Immunohistochemical expression of epidermal growth factor receptor (EGFR) in canine mammary tissues. *Res Vet Sci* 87:432–437
50. Rakha EA, El-Sayed ME, Green AR, Paish EC, Lee AH, Ellis IO (2007) Breast carcinoma with basal differentiation: a proposal for pathology definition based on basal cytokeratin expression. *Histopathology* 50:434–438
51. Wolff AC, Hammond ME, Hicks DG, Dowsett M, McShane LM, Allison KH et al (2014) Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: american Society of Clinical Oncology/College of American Pathologists clinical practice guideline update. *Arch Pathol Lab Med* 138:241–256
52. Bravaccini S, Granato AM, Medri L, Foca F, Falcini F, Zoli W et al (2013) Biofunctional characteristics of in situ and invasive breast carcinoma. *Cell Oncol (Dordr)* 36:303–310
53. Campos LC, Silva JO, Santos FS, Araújo MR, Lavalle GE, Ferreira E et al (2015) Prognostic significance of tissue and serum HER2 and MUC1 in canine mammary cancer. *J Vet Diagn Invest* 27:531–535
54. Beha G, Brunetti B, Asproni P, Muscatello LV, Millanta F, Poli A et al (2012) Molecular portrait-based correlation between primary canine mammary tumor and its lymph node metastasis: possible prognostic-predictive models and/or stronghold for specific treatments? *BMC Vet Res* 8:219
55. Dutra AP, Granja NV, Schmitt FC, Cassali GD (2004) c-erbB-2 expression and nuclear pleomorphism in canine mammary tumors. *Braz J Med Biol Res* 37:1673–1681
56. Kim JH, Im KS, Kim NH, Yhee JY, Nho WG, Sur JH (2011) Expression of HER-2 and nuclear localization of HER-3 protein in canine mammary tumors: histopathological and immunohistochemical study. *Vet J* 189:318–322
57. Muhammadnejad A, Keyhani E, Mortazavi P, Behjati F, Haghdoust IS (2012) Overexpression of her-2/neu in malignant mammary tumors; translation of clinicopathological features from dog to human. *Asian Pac J Cancer Prev* 13:6415–6421
58. Singer J, Weichselbaumer M, Stockner T, Mechtcheriakova D, Sobanov Y, Bajna E et al (2012) Comparative oncology: erbB-1 and ErbB-2 homologues in canine cancer are susceptible to cetuximab and trastuzumab targeting. *Mol Immunol* 50:200–209
59. Im KS, Kim NH, Lim HY, Kim HW, Shin JI, Sur JH (2014) Analysis of a new histological and molecular-based classification of canine mammary neoplasia. *Vet Pathol* 51:549–559
60. de las Mulas JM, Ordás J, Millán Y, Fernández-Soria V, y Cajal SR (2003) Oncogene HER-2 in canine mammary gland carcinomas: an immunohistochemical and chromogenic in situ hybridization study. *Breast Cancer Res Treat* 80:363–367
61. Burrai GP, Tanca A, De Miglio MR, Abbondio M, Pisanu S, Polinas M et al (2015) Investigation of HER2 expression in canine mammary tumors by antibody-based, transcriptomic and mass spectrometry analysis: is the dog a suitable animal model for human breast cancer? *Tumour Biol* 36:9083–9091
62. Liu D, Xiong H, Ellis AE, Northrup NC, Rodriguez CO Jr, O'Regan RM et al (2014) Molecular homology and difference between spontaneous canine mammary cancer and human breast cancer. *Cancer Res* 74:5045–5056
63. van de Vijver MJ (2014) Molecular tests as prognostic factors in breast cancer. *Virchows Arch* 464:283–291