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Effect of Ovariohysterectomy at the Time of Tumor Removal in Dogs with Mammary Carcinomas: A Randomized Controlled Trial

V.M. Kristiansen, L. Peña, L. Díez Córdova, J.C. Illera, E. Skjerve, A.M. Breen, M.A. Cofone, M. Langeland, J. Teige, M. Goldschmidt, and K.U. Sørenmo

Background: Ovarian hormones play crucial roles in mammary carcinogenesis. However, whether ovarian ablation by ovariohysterectomy (OHE) improves the prognosis in dogs with mammary carcinomas is unclear.

Objectives: Determine if OHE at the time of mastectomy improves the prognosis in dogs with mammary carcinomas and evaluate if hormonal factors influence the effect of OHE.

Animals: Sixty intact dogs with mammary carcinomas.

Methods: Dogs were randomly assigned in a 1 : 1 ratio to undergo OHE (n = 31) or not (n = 29) at the time of tumor removal. Peri-surgical serum estradiol (E2) and progesterone concentrations were measured, tumor diagnosis was confirmed histologically, and tumor estrogen and progesterone receptor status was immunohistochemically determined. The dogs were monitored for recurrence and metastases every 3-4 months for at least 2 years. Uni- and multivariable survival analyses were performed with relapse and all-cause death as endpoints in addition to univariable subgroup analyses.

Results: Overall, OHE did not significantly decrease hazard of relapse (hazard ratio [HR], 0.64; $P = .18$) or all-cause death (HR, 0.87; $P = .64$) in univariable analyses. In multivariable analysis OHE did not significantly influence the hazard of relapse (HR, 0.54; $P = .12$), but an interaction effect was identified between ER status and E2 ($P = .037$). Subgroup analysis identified decreased hazard of relapse in the OHE group compared to the non-OHE group in the subsets of dogs with increased E2 (HR, 0.22; $P = .012$) or grade 2 tumors (HR, 0.26; $P = .02$).

Conclusion: Dogs with grade 2, ER-positive tumors, or with increased peri-surgical serum E2 concentration represent a subset of dogs with mammary carcinomas likely to benefit from OHE.

Key words: Cancer; Canine; Malignant tumors; Spaying.

Mammary carcinomas are the most common malignancies in intact female dogs and as such represent a substantial health problem in this population.[1–6]

Abbreviations:

CMT	canine mammary tumor
OHE	ovariohysterectomy
HoR	hormone receptor
IHC	immunohistochemistry
DFP	disease-free period
OS	overall survival
P4	progesterone
E2	17β-estradiol
ER	estrogen receptor
PR	progesterone receptor
PS	percentage of stained cells
IS	staining intensity
TS	total score
PI	proliferation index
FNA	fine needle aspiration
HD	histological diagnosis
g	gamma
HR	hazard ratio

Surgery remains the standard of care, and dogs with early stage, low-grade tumors often are treated effectively by surgery alone. Metastatic disease and tumor recurrence, however, are common causes of failure in dogs with more advanced or higher grade disease, indicating a need for additional treatment for this subset of patients.[7,8]

Ovarian hormones and their respective receptors in mammary tissue have long been recognized as key factors in the development of canine mammary tumors (CMTs).[9] An association between ovarian hormonal exposure and risk for mammary tumors has been suggested by epidemiological studies reporting significantly decreased incidence of mammary tumors in dogs having

From the Faculty of Veterinary Medicine and Biosciences, Norwegian University of Life Sciences, Oslo, Norway (Kristiansen, Skjerve, Breen, Langeland, Teige); the Facultad de Veterinaria, Universidad Complutense de Madrid, Madrid, Spain (Peña, Díez Córdova, Illera); the Veterinary Specialty Center of Delaware, New Castle, DE (Cofone); and the School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA, USA (Goldschmidt, Sørenmo). The clinical and routine histology parts of the study were carried out at the School of Veterinary Science, Norwegian University of Life Sciences, Norway. Immunohistochemistry and serum hormone analyses were performed at Universidad Complutense de Madrid, Spain.

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Corresponding author: V.M. Kristiansen, Department of Companion Animal Clinical Sciences, Faculty of Veterinary Medicine and Biosciences, Norwegian University of Life Sciences, P.O. Box 8146 Dep., N-0033 Oslo, Norway; e-mail: veronica.kristiansen@nmbu.no.

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undergone prepubertal ovariectomy or ovariohysterectomy (OHE) compared to intact dogs or dogs ovarioectomized later in life[10,11] A protective effect of OHE is supported by the lower incidence of CMTs seen in countries where routine early-age spaying is common,[2,3] compared to countries where it is not, as in several European countries including Norway.[1,4–6] Although a recent systematic review[12] regarded the scientific level of these studies too weak to draw a firm conclusion, it is biologically likely that ovarian hormones are important in the etiology of mammary tumor development. Many of these tumors express hormone receptors (HoRs), which suggests some degree of hormone dependency[13–15] that may be exploited in treatment. In contrast to breast cancer in humans in whom hormonal treatment is effective in patients with HoR-positive tumors, this remains controversial in the dog. Several studies, predominantly retrospective, have been conducted to answer this question, but with contradictory results. The majority of these studies did not find a prognostic benefit of OHE in dogs with mammary tumors, apart from 2 studies.[16–21] None of these, however, evaluated the effect of OHE in the context of the tumors' HoR profiles. This may in part explain the negative results. Similar to women, only dogs with HoR-positive tumors are likely to benefit from hormonal ablation.

Complicating this issue is the fact that HoR analyses are not routinely performed in CMT because the immunohistochemical (IHC) methods of HoR labeling are not standardized or routinely available outside specific research projects.[22] Furthermore, the predictive value of response to OHE has never been evaluated in CMT, nor have the cut-off values for establishing positive versus negative results. Moreover, the normal mammary gland undergoes dynamic histological and HoR changes throughout the estrous cycle.[23] The HoR status of the tumor has been found to be affected by the estrous cycle and spay status in addition to factors such as age, tumor size, and histological features.[9,14,23,24] Some of these characteristics might provide practical measures to predict if an individual dog will benefit from OHE or not.

The purpose of our study was to evaluate the effect of OHE on disease-free period (DFP) and overall survival (OS) in dogs with mammary carcinomas using a randomized controlled trial (RCT) design. Additionally, the relevance of hormonal factors to this effect was investigated in order to identify practical predictive markers for response to OHE.

Materials and Methods

Study Population and Clinical Assessment

The study was designed as a RCT involving intact female dogs with histologically confirmed mammary carcinomas without distant metastases, other serious diseases, or any previous history of mammary malignancy. A sample size calculation was performed[25] which estimated that a minimum of 60 dogs with mammary carcinoma must be included to detect a 30% difference in DFP between the OHE group and the non-OHE group using a *P*-value of .05 and power of 80%. A complete reproductive-, tumor-, and health history was collected as part of the screening and initial enrollment. For further consideration for inclusion, the owners had to sign a written consent form in which they agreed to the randomization procedure. The study was approved by the institutional animal care and use board at the Norwegian School of Veterinary Science (Oslo, Norway).

The dogs underwent complete physical examination and staging consisting of CBC, serum biochemistry profile, urinalysis, cytological evaluation of enlarged draining lymph nodes, and 3-view thoracic radiographs. All tumors were recorded (number, localization) and size was determined as the largest diameter measured by caliper. Clinical stage was determined using the modified World Health Organization (WHO) classification system[26] with stage 1, 2, and 3 indicating local disease with increasing tumor size (T1, <3 cm; T2, 3–5 cm; T3, >5 cm), stage 4 representing dogs with lymph node metastasis regardless of tumor size, and stage 5 constituting distant metastases (excluded from the present study). An additional blood sample was collected from each dog at the time of staging and serum was decanted and stored (−20°C) until later assessment of steroid hormone concentrations (progesterone, [P4] and 17β-estradiol, [E2]).

Randomization and Treatment Protocol

Before surgery, dogs were randomly assigned in a 1 : 1 ratio to undergo either tumor removal with concomitant OHE (OHE group) or tumor removal only (non-OHE group). The dogs were stratified based on tumor size (<3, ≥3 cm) and age (<9, ≥9 years) to ensure equal distribution of these 2 prognostic factors between the treatment groups. A block randomization scheme was used within each stratum. The allocation sequence was computer generated, and not known to owners or the investigator until the informed consent was signed. There was 1 fixed cost for the owner regardless of the type of mammary tumor surgery or whether OHE was performed or not. Surgery was performed according to standard surgical principles[7,27,28] and involved excision of all tumors with margins. The size and extent of surgery depended on the size and numbers of tumors. Dogs with stage 4 disease had the affected lymph node resected en bloc together with the mammary tumor whenever possible. All dogs with tumors in the inguinal or caudal abdominal mammary glands had both glands removed together with the ipsilateral inguinal lymph node. The OHE was performed at the time of tumor surgery or within 2 weeks after tumor removal if the size or extent of tumors required the surgical treatment to be staged. Only dogs with at least 1 carcinoma were included in this study. The enrollment of dogs thus continued until the required number of 60 dogs was reached. The outcome in the dogs with only benign tumors has been reported previously.[29]

Histopathological Examination

Resected tumors and lymph nodes were fixed in 10% buffered formalin and routinely processed for histopathology. The slides were evaluated independently by 2 pathologists (M. G. and J. T.) and classified according to the type of tissue present (epithelial, myoepithelial, connective tissue, or some combination of these) and whether they were malignant or benign. Only dogs for which both pathologists agreed on the presence of at least 1 malignant epithelial tumor were included in this study. These were further diagnosed and graded (by L. P. and M. G.) according to the proposed CMT classification[30] and the recent proposed grading system especially adapted to CMT.[31] The tumor resection margins were evaluated histologically and reported as incomplete if tumor cells were present at the edge of resected tissue. Scar revision was recommended in dogs with incomplete margins if feasible and appropriate (ie, adequate tissue and localized disease). In dogs

with >1 carcinoma, the largest or highest grade tumor was selected for further analyses.

Immunohistochemistry

Immunohistochemistry (IHC) of estrogen receptor alpha (ER), progesterone receptor (PR), and Ki-67 (a proliferation marker) performed on the selected carcinomas at the Laboratory of Pathology, Veterinary Clinical Hospital at the Veterinary School of the Complutense University of Madrid (HCVC), Spain. Briefly, paraffin sections were placed in a pretreatment module[a] containing EDTA buffer solution (pH 8.0)[b] for 20 minutes at 95°C, cooled down to 60°C, and rinsed in warm tap water. An automatic Immunostainer device[a] was used for immunostaining. Commercially available clones (incubated 90 min at room temperature) and detection system kits were used for antigen labeling: for ER clone 1D5[c] (diluted 1/10) and a developing system[d]; for PR clone Mab 1E2[e] prediluted) and for Ki-67 clone SP6[f] (prediluted) and a kit detection system.[g] After immunostaining the slides were counterstained with hematoxylin and permanently mounted with DPX.[h] Negative control samples consisted of tissue sections in which the primary antibody was replaced with a nonreactive antibody (mouse serum for ER and PR and rabbit serum for Ki-67). Positive controls consisted of tissue sections from human and CMT with previously demonstrated reactivity to the respective primary antibody and also consisted of internal tissue controls. Receptor labeling within nuclei was evaluated by counting ≥1,000 tumor epithelial cells in 8–10 microscopic fields (40× magnification) representative of histological type and grade. Stromal cells were not considered.

Scoring of ER, PR and Ki-67 Proliferation Index

Scoring of ER- and PR immune staining was done using the Allred method.[22,32] In this method both the percentage of stained cells (PS): none = 0, <1% = 1, 1–10% = 2, 10–33% = 3, 33–66% = 4, 66–100% = 5) and the staining intensity (IS): absent = 0, weak = 1, moderate = 2, and strong = 3) are measured and summarized as a total Allred score (TS). The total score (PS + IS) was equal to 0 or ranged from 2 to 8. Labeling was considered uninterpretable or unsuccessful when no tumor nuclei were immunoreactive (with internal positive control lacking nuclear labeling).[22] Ki-67 proliferation index (PI) was expressed as percentage of Ki-67 immune-stained tumor cells related to the total number of cells determined by counting positive and negative nuclei of tumor cells in 8–10 representative fields. Ki-67-stained nuclei were considered positive regardless of staining intensity.

Serum Hormone Analyses

Serum samples collected on or close to the day of surgery were analyzed for content of E2 and P4 with concentration expressed in pg/ml for E2 and ng/ml for P4. All samples were assayed at the Laboratory of Pathology of the SVTCU using competitive enzyme immunoassay.[33]

Postoperative Follow-Up

Postoperative clinical evaluations were scheduled every 3–4 months for 2 years, and every 4–6 months thereafter until death, censoring, or end of study. During these visits, local recurrences and new tumors were recorded and characterized (number, size, localization, and invasiveness) together with metastases as determined by the restaging procedure (fine needle aspirates [FNA] from enlarged lymph nodes and 3-view thoracic radiographs at a minimum). Other health problems also were recorded. For a mammary mass to be classified as a new tumor it had to be at least 1 mammary gland away from the original tumor or, if closer, the original tumors had to have had histologically complete margins. A second tumor excision was recommended in these dogs and the diagnosis reviewed if the tumor was removed. Local recurrence was defined as tumors located close to the site where the original tumor was removed. Complete necropsy was requested for all dogs that died or were euthanized during the study period. Cause of death or euthanasia was registered and classified as not mammary tumor-related or mammary tumor-related. In order for death to be classified as the latter, the owner's decision to euthanize had to be directly related to mammary tumor disease confirmed by cytology, histology, or diagnostic imaging. The time period from surgery to any type of disease progression or death was recorded. If a dog died or a tumor event was detected between regular reevaluations, the accurate time of death or the time the owner first noted the tumor event was used.

Clinical End Points

The primary endpoints were DFP defined as the time period from surgery to detection of relapse (new tumor, local recurrence, or metastases), and OS defined as the time period from surgery to death from all causes. In dogs experiencing >1 tumor event, the DFP was determined by the event that occurred first.

Explanatory Variables

In addition to the intervention variable (spayed or not at the time of tumor removal: OHE versus non-OHE), the following pre- and postoperative variables were investigated for possible influence on outcome: age, tumor size (WHO categories), number of tumors (solitary versus multiple), number of malignant tumors (solitary versus multiple), clinical stage (WHO categories), type of surgery (local versus extensive), tumor margins (histologically complete versus incomplete), carcinoma subtypes, tumor grade, serum P4 (low versus high), serum E2 (low versus high), Ki-67 PI (low versus high), tumor ER- and PR expression (negative versus positive).

The carcinoma subtypes were categorized in 3 histological diagnoses (HD) groups: HD1, HD2, and HD3 following criteria previously described[31] in which HD1 consists of in situ carcinoma, simple carcinoma, carcinoma arising in a mixed tumor, complex carcinoma, mixed type carcinoma, ductal carcinoma, or adenosquamous carcinoma; HD2 of solid carcinoma, comedocarcinoma, or anaplastic carcinoma; whereas HD3 included other histological types. In dogs with multiple carcinomas, the tumor with the most severe clinicopathologic feature was chosen for further analyses. The concentration of E2 in serum was categorized using the median value as cut-off because there is little knowledge of how E2 relates to specific stages in the estrous cycle,[34] and because of the assumption that it is not normally distributed. The concentration of P4 in serum was categorized using 1.3 ng/mL as a cut-off because this value is suggested to discriminate dogs in anestrus from dogs in luteal phase.[35] The threshold for ER- and PR-positive tumors was based on sensitivity and specificity analyses using different cut-off values for Allred score for the respective HoR status evaluated against clinical outcome.[22] The cut-off with maximum sensitivity and specificity was selected. Similarly, the cut-off for Ki-67 PI positivity was determined based on how it related to clinical outcome in this dataset.

Statistical Analyses

For each of the explanatory variables, the OHE group was compared with the non-OHE group using nonsignificant chi

square test results with the *P*-value as an indicator of similar group characteristics. The number of dogs experiencing any mammary tumor event, median time in months to the event or censoring, and total number of dogs in each treatment group were calculated. Dogs lost to follow-up or still alive without disease progression were censored at the date of last known status. Dogs that developed diseases requiring OHE were censored at the date of that surgery. The Kaplan–Meier method was used to calculate estimates of DFP and OS with treatment differences assessed by the use of log-rank test.

The OHE status and the other investigated variables were evaluated by univariable Cox proportional hazard models for their effect on DFP and OS. Time at risk was defined as months from the date of surgery to the event of interest or censoring. The Efron approximate method was used in this analysis. In order to investigate if factors biologically assumed to be associated with HoR positivity influenced the dogs' response to OHE, prespecified subgroup analyses constituting the variables ER, PR, E2, P4, Ki-67 PI, and grade were performed. For each variable, the effect of OHE on DFP was investigated in each subcategory by the use of Cox proportional hazard models.

In order to evaluate the effect of OHE on outcome, while adjusting for other explanatory variables, multivariable Cox proportional hazards models were constructed. Whether to use DFP, OS or both as endpoints in these depended on how OHE performed in the univariable analysis. Explanatory variables were offered to the multivariable models based on their statistical significance level in the univariable analysis ($P < .20$), their biological relevance as determined a priori, and based on the results of collinearity tests. Collinearity between categorical variables was evaluated using Goodman and Kruskals gamma (g) with associations >0.7 and <−0.7 considered as evidence of collinearity. The multivariable model was built using manual forward selection, and the predictors were retained in the final model if $P < .05$ or if assumed to be biologically relevant. Confounding and intervening variables were considered based on both a tentative causal diagram and changes in effects of the other variables in the models. Biologically plausible 2-way interactions were evaluated by insertion of an interaction factor in the models and kept if $P < .05$. Thus possible interactions were tested between OHE status and hormonal factors and also among different hormonal factors if considered in the same model. Survival curves were generated based on estimates from the models. The assumption of proportional hazards was evaluated based on Schoenfeld residuals for the variable OHE in the model. Potential outlying observations with influence on the model were evaluated as previously described.[36] All analyses were performed using the software package Stata.[i]

Results

Enrollment and Baseline Characteristics

Sixty of 330 dogs assessed for eligibility were included between January 2005 and May 2011, with 31 dogs allocated to the OHE group and 29 to the non-OHE group. Figure 1 outlines the enrollment process. Reproductive and tumor history were known for all dogs. Fifty-one dogs (82%) were nulliparous. Four dogs (6.4%) had history of hormone treatment (estrus control) and 5 dogs (8.1%) had benign mammary tumors previously removed. Table 1 provides an overview of all baseline dog and tumor characteristics sorted by treatment arm. The distribution of characteristics across study groups was well balanced as reflected by no significant differences (Table 1).

Immunostaining for PR and ER was successful in 56 and 52 tumors, respectively, of the 60 selected carcinomas. The threshold for both PR and ER positivity was

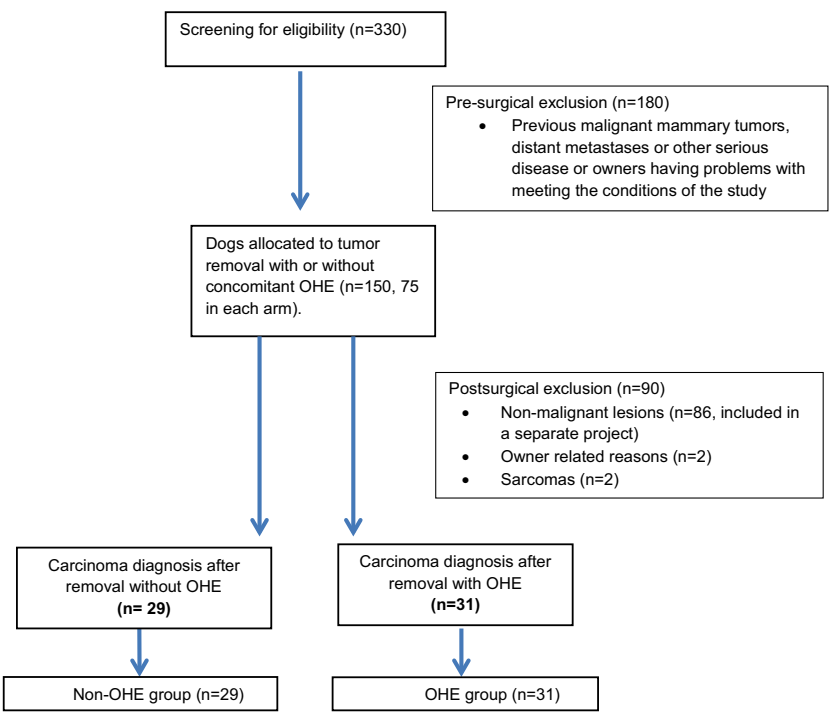

Fig 1. Flow diagram of the enrollment and randomization in a randomized-controlled trial investigating the effect of ovariohysterectomy (OHE) at the time of tumor removal in 60 dogs with mammary carcinomas.

Table 1. Description of baseline characteristics in 60 dogs with mammary carcinomas randomized to ovariohysterectomy (OHE group) or to remain intact (non-OHE group) during tumor removal. Similar group characteristics are verified by a nonsignificant *P*-value (>.05) using chi square test on categorized variables.

	Non-OHE Group (n = 29)	OHE Group (n = 31)	*P*-Value
Age mean (SD)	9.53 (0.3)	9.56 (0.3)	
≥9.0 years	15	14	.61
<9.0 years	14	17	
Bodyweight mean (SD)	24.4 (2.3)	21.1 (1.5)	
>10 kg	22	26	.44
≤10 kg	7	5	
Breed			
Pure[a]	23	18	.077
Mixed	6	13	
Duration[b]			
≥6 months	12	18	.20
<6 months	17	13	
Multiple malignant tumors[c]			
Yes	9	5	.17
No	20	26	
Tumor size, mean (SD)	4.0 (2.03)	4.6 (2.58)	
≥3 cm	21	22	.90
<3 cm	8	9	
Tumor size			
<3 cm	8	9	.98
3–5 cm	13	13	
>5 cm	8	9	
Clinical stage			
1 (T < 3 cm, N0, M0)	8	9	.63
2 (T3–5 cm, N0,M0)	11	10	
3 (T > 5 cm, N0, M0)	6	10	
4 (T any size, N1, M0)	4	2	
Type of surgery			
Local surgery[d]	9	8	.65
Extensive surgery[e]	20	23	
State of margins			
Complete	27	28	.70
Incomplete[f]	2	3	
Carcinoma subtypes[g]			
HD1	20	18	.66
HD2	8	11	
HD3	1	2	
Tumor grade[h]			
1	15	10	.29
2	7	12	
3	7	9	
Clinical anestrus			
Yes	20	19	.53
No	9	12	
P4			
<1.3 ng/mL	19	17	.85
≥1.3 ng/mL	8	8	
E2			
<35 pg/mL	13	13	.78
≥35 pg/mL	14	12	
ER[i]			
Neg	6	10	.31
Pos	19	17	
PR[i]			
Neg	4	6	.57
Pos	23	23	
Ki-67 PI			
≤20	19	21	.93
>20	6	7	

PI, proliferation index.

[a]28 different breeds.

[b]Time from the tumor was first discovered by owner to the time of project inclusion.

[c]12 dogs had 2 malignant tumors and 3 dogs had 3 tumors.

[d]Lumpectomy or simple mastectomy.

[e]Regional or radical mastectomy.

[f]Only 2 of these dogs were reoperated. The other were not because of the aggressive nature of the removed tumors.

[g]Grouping of carcinomas was performed using criteria previously published by Pena et al.[31]; HD1 includes in situ carcinoma (1), simple carcinoma (12), carcinoma arising in a mixed tumor (4), complex carcinoma (13), mixed carcinoma (8), ductal carcinoma (1), and adenosquamous carcinoma (1. HD2 includes solid carcinoma (10), comedocarcinoma (4), carcinoma and malignant myoepithelioma (3), and anaplastic carcinoma (2). HD3 includes other histological types here represented by lipid-rich (secretory) carcinoma (1), carcinosarcoma (1), squamous cell carcinoma (1).

[h]Grading of the tumors was determined using the grading system proposed by Pena et al.[31]

[i]A positive tumor estrogen- or progesterone receptor status was determined by an Allred score ≥3.

set to Allred TS ≥ 3. According to this categorization, 46 tumors (82%) were PR-positive, 36 tumors (69%) ER-positive, and 49/56 tumors (88%) positive for PR, ER, or both. Hormonal receptor status according to subtypes of carcinomas and treatment group is shown in Table 2. The Ki-67 PI was successfully measured in 53 of the 60 carcinomas and ranged from 1.2 to 75.8%. A Ki-67 PI >20% was considered as the cut-off for high Ki-PI. After this categorization, the Ki-67 PI was low in 13 tumors and high in 40 tumors.

Dogs with PR-positive tumors were associated with high E2 ($P = .002$), low PI ($P = .002$), grade 1 ($P = .007$ compared to both grades 2 and 3), and HD group 1 ($P = .013$). Dogs with ER-positive tumors were associated with grade 1 ($P = .000$ compared to grade 3) and grade 2 tumors ($P = .019$ compared to grade 3). Number of tumors in each of these categories is presented in Table 3. Associations indicated in this table were reflected by strong positive correlations between PR and E2 (g = 1.0), ER and PR (g = 0.79) and grade and PI (g = 0.81) and also by the strong negative correlation between PR and PI (g = −0.82) and between PR and grade (g = −0.77) identified by the collinearity analysis.

Table 2. Distribution of dogs based on carcinoma subtype, treatment allocation, and hormonal receptor status (estrogen receptor, ER; progesterone receptor, PR) in a randomized study investigating effect of concomitant ovariohysterectomy (OHE) in the treatment of 60 dogs with canine mammary carcinomas; 31 dogs had OHE and 29 remained intact during tumor removal.

		Treatment Allocation		Hormone Receptor Status	
Histological Diagnosis (HD)	Total No of Tumors	non-OHE	OHE	ER+	PR+
HD1					
Simple carcinoma	11	6	5	9	10
Carcinoma arising in a mixed tumor	4	1	3	3	4
Complex carcinoma[a]	13	7	6	9	11
Mixed carcinoma[b]	8	5	3	5	6
Ductal carcinoma	1	0	1	1	1
Adenosquamous carcinoma	1	1	0	0	1
HD2					
Solid carcinoma[c]	10	5	5	4	6
Comedocarcinoma	4	1	3	1	2
Carcinoma and malignant myoepitelioma	3	0	3	0	1
Anaplastic carcinoma	2	2	0	1	1
HD3					
Lipid-rich (secretory) carcinoma	1	0	1	1	1
Carcinosarcoma	1	1	0	1	1
Squamous cell carcinoma	1	0	1	1	1
Total	60	29	31	36	46

[a]4/13 ER and 2/13 PR results were missing.
[b]2/8 ER results were missing.
[c]2/10 ER and 2/10 PR results were missing.

Table 3. Relationship between hormone receptor expression, tumor characteristics, and circulating hormones in 60 dogs with mammary carcinomas. The dogs participated in a randomized-controlled trial investigating the effect of OHE.

Variable	No. of Tumors	ER +	ER−	PR +	PR −
Serum E2					
+	26	17	5	26	0
−	26	13	9	15	7
Serum P4					
+	16	10	3	13	1
−	36	20	11	28	6
Ki-67 PI					
≤20	40	28	9	36	3
>20	13	7	6	7	6
Grade					
1	25	20	1	24	0
2	19	11	4	12	4
3	16	5	11	10	6
HD					
1	38	27	5	33	3
2 + 3	22	9	1	13	7

Follow-Up and Clinical Outcome

All 60 dogs were available for regular follow-up until death or censoring with a median follow-up time of 18.5 months (range 0.5–76) for the OHE group and 22 months (range 1–61) for the non-OHE group. Except for 1 dog in the non-OHE group that was spayed later because of pyometra (censored at that date), none of the dogs received any other treatment during the study period. Two dogs (1 in each treatment group) were lost to follow-up, but contributed to the study until censoring at the date of last evaluation. All evaluations were performed (by K. U. S. or V. M. K.) at the School of Veterinary Medicine, Oslo, Norway until May 2013.

Relapse occurred in 18 dogs (58%) in the OHE group and 21 dogs (72%) in the non-OHE group with a median DFP of 13.5 months (range, 0.5-75.9) for the OHE group and 11.5 months (range 0.9–44) for the non-OHE group. Number of dogs and time to each type of relapse (new tumor, local recurrence, regional, and distant metastases) are outlined according to treatment group in Table 4.

Death occurred in 50 dogs (83%) by the end of the study; 27 dogs (87%) in the OHE group and 23 dogs (79%) in the non-OHE group. Ten dogs (17%) were still alive; 4 in the OHE group and 6 in the non-OHE group. Median OS was 21 months (range 0.5–76) for the OHE group and 23.7 months (range 0.9–60) for the non-OHE group. Neither DFP nor OS differed significantly between the treatment groups. Necropsy was performed in 43/50 dogs. Twenty-six dogs (42%), 13 in each treatment group died because of mammary cancer. More specifically, 24 dogs died from distant metastasis and 2 dogs because of large or ulcerated new mammary tumors. Distant metastases were confirmed by necropsy in all but 2 dogs in which metastases were diagnosed by imaging. The most common site for metastasis was the lungs (n = 21), but 15 dogs also had metastases in ≥1 of the following organs: distant lymph nodes (n = 14), adrenal gland (n = 1), skin (n = 5), liver (n = 3), pleura/

Table 4. Number of dogs and time to different types of relapse in 60 dogs after tumor removal combined with (n = 31) or without (n = 29) ovariohysterectomy (OHE) in a randomized study investigating effect of OHE on prognosis in dogs with mammary carcinoma.

Type of Postoperative Event[a]	OHE-Status	Number of Dogs with the Event	Median Time to the Event (Months)	Median Time to Censoring (Months)
New tumor	Non-OHE	13	10.8	22.0
n = 23 (38%)	OHE	10	9.6	30.8
Local recurrence	Non-OHE	7	8.0	24.5
n = 14 (23%)	OHE	7	5.4	36.8
Distant	Non-OHE	11	8.0	25.2
metastases	OHE	12	13.0	39.3
n = 23 (38%)				
Regional	Non-OHE	7	8.0	25.2
metastases	OHE	8	9.2	34.3
n = 15 (25%)				

[a]Several dogs had more than 1 tumor event. In the survival analysis disease-free period was terminated by the first occurring of these tumor events.

diaphragm (n = 3), kidney (n = 5), urinary bladder (n = 2), myocardium (n = 1), muscle (n = 1), and vertebra (n = 2).

Univariable and Subgroup Analysis

The OHE variable was not found to be statistically significant in the univariable screening for either DFP (hazard ratio [HR], 0.64; 95% confidence interval [CI], 0.33–1.22; $P = .19$) or OS (HR, 0.87; 95% CI, 0.49–1.54; $P = .64$). The Kaplan–Meier survival curves are shown in Fig 2.

The results of the prespecified subgroup analyses regarding effect of OHE stratified on HoR status, perisurgical concentration of serum E2, grade, and Ki-67 status is depicted by Kaplan–Meier survival curves in Fig 3. In the subset of dogs with ER-positive tumors, the HR of relapse between the OHE versus non-OHE group was 0.5, but this did not reach significance, $P = .11$, see Fig 3A. No difference in the hazard of relapse was noted between the treatment groups in dogs with ER-negative tumors (HR, 1.6; $P = .46$). The same pattern was observed when stratification was performed on PR status (PR-positive tumors: HR, 0.56, $P = .15$; PR negative tumors: HR, 0.7, $P = .6$) and also when dogs were stratified on Ki-67 PI (low PI: HR, 0.47, $P = .093$; high PI: HR, 1.7, $P = .42$). Interestingly, when dogs were stratified according to serum E2 (high versus low) at the time of surgery, OHE was strongly protective in dogs with high E2, but had no effect in dogs with low E2 (high E2: HR, 0.22; $P = .012$; low E2: HR, 1.5; $P = .39$). When effect of OHE was stratified based on tumor grades, dogs with grade 2 tumors had significant improvement in DFP if assigned to the OHE group compared to intact dogs. No difference was observed between the treatment groups in dogs with grade 1 or grade 3 tumors (grade 1, $P = .52$; grade 2, $P = .02$; grade 3, $P = .39$).

Multivariable Analysis

Multivariable analysis was performed with DFP as the outcome variable because univariable testing indicated some influence of OHE on DFP ($P < .2$) but not on OS ($P \gg .2$). Variables considered in addition to OHE for inclusion in the multivariable analysis based on collinearity test and P-level in the univariable model were state of margins (complete margins versus uncomplete: HR, 0.048; $P = .00$), Ki-67 PI (high PI versus low PI: HR, 3.5; $P = .00$) HD-group (HD2 versus HD1: HR, 2.97; $P = .002$; HD3 versus HD1: HR, 0.62; $P = .64$), grade (grade 2 versus grade 1: HR, 1.06; $P = .88$; grade 3 versus grade 1: HR, 2.8; $P = .008$), stage (stage 3–4 versus stage 1–2: HR, 1.9; $P = .047$), surgery type (extensive versus local: HR, 1.75; $P = .16$), E2 (high versus low: HR, 0.53; $P = .073$), and PR status (negative versus positive: HR, 0.5; $P = .094$). The stratification variables age and size were not found to be statistically significant in the univariable screening and did not appear as confounders, and therefore were omitted from further model building. Because many variables had some degree of correlation they could not be included in the same model. Therefore, 3 models were made; 2 consisting of OHE and tumor factors and 1 model consisting of OHE and hormone factors (Table 5). Grade 3 was found to be a stronger predictor for relapse than OHE (HR, 2.8; $P = .007$). Likewise, the effect of OHE was almost eradicated when the effect of Ki-67 PI was added to the model (Table 5). In the model based on OHE and hormone factors, a significant interaction effect was identified between ER and E2 ($P = .037$). This interaction indicated increased protective effect of OHE in dogs with ER-positive disease and high serum estrogen concentration as compared to dogs in which these variables were negative or low (Table 5). In Fig 4, these interactions are illustrated by DFP survival curves generated from this multivariable model. Intact dogs with low E2 (E2-) and ER-negative tumors (bottom curve) represent the subgroup with shortest DFP and can be considered as baseline. Performing OHE in this subgroup was associated with only slight improvement in DFP. In contrast, dogs with both positive ER status and high E2 benefited from OHE as indicated by the upper curve in Fig 4.

Discussion

Overall, our study found no significant benefit in DFP or OS from ovarian hormonal ablation in dogs with mammary carcinomas. However, mammary carcinomas in dogs represent a histologically and biologically heterogeneous group of diseases and the findings must be interpreted in light of this diversity. Biologically, it is plausible that only dogs with positive HoR status would benefit from OHE because the effect of ovarian hormones is mediated through HoRs. When the effect of OHE was investigated in separate subgroups stratified

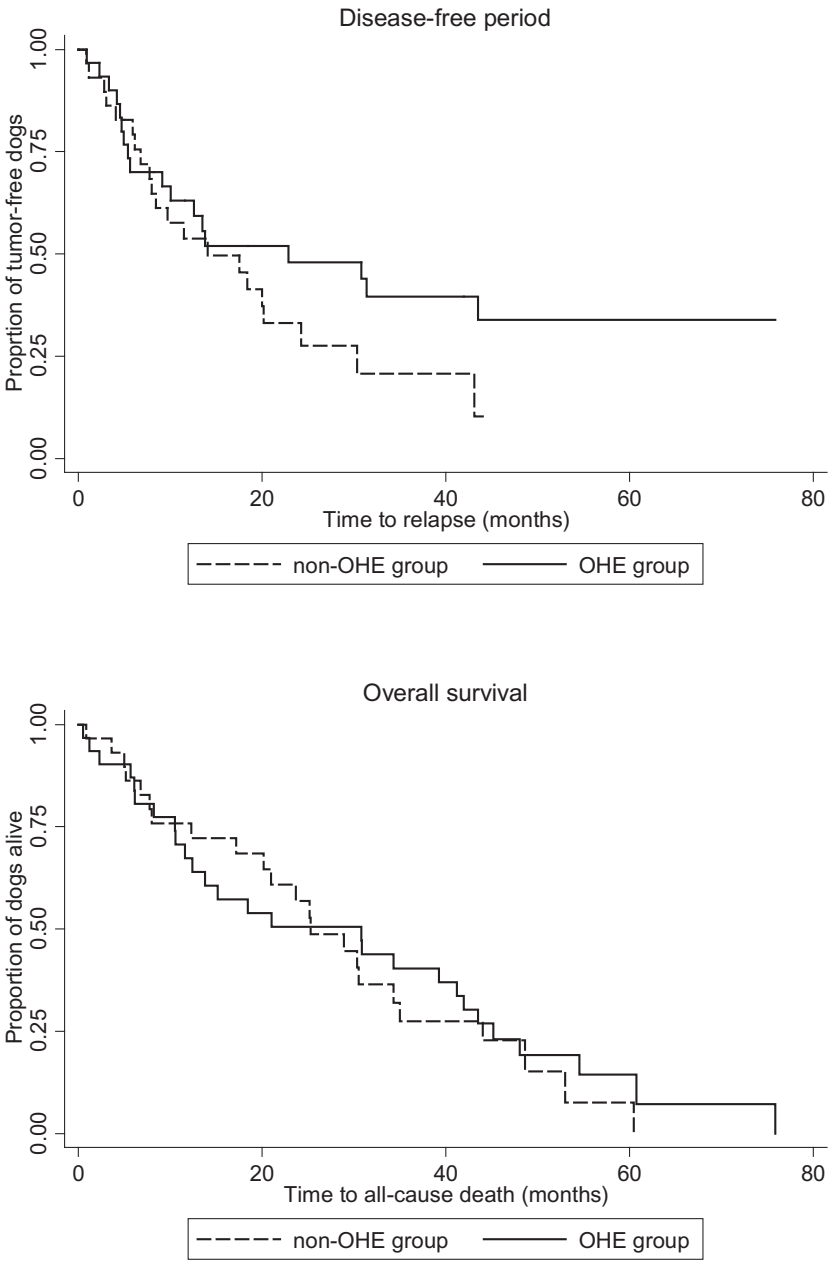

Fig 2. Kaplan–Meier curves depicting disease-free period (failures = 39, $P = .18$) and overall survival (failures = 50, $P = .64$) in 60 dogs with mammary carcinoma participating in a randomized controlled trial investigating the prognostic effect of ovariohysterectomy (OHE) performed at the time of tumor removal. Thirty-one dogs underwent OHE at the time of tumor removal while 29 dogs remained intact (solid line, OHE-group; dashed line, non-OHE group).

by tumor ER expression, grade, Ki-67 PI and serum E2, OHE conferred a significant benefit in dogs with high E2 ($P = .012$) and dogs with grade 2 tumors ($P = .02$). Contrary to our hypothesis, the protective effect seen from OHE in dogs with ER-positive tumor and dogs with low Ki-67 expression did not reach significance with P values of .11 and .093, respectively. These results are suggestive of an association between tumor HoR and response to hormonal treatment and consistent with reports of breast cancer in humans.[37,38]

Results from the subgroup analysis must be interpreted with caution because the prior power calculation was based on the entire population. The analyses of effect of OHE in sub-groups therefore lack satisfactory statistical power. Also, the subgroup analyses were univariable and thus unadjusted for other variables. The results derived from subgroup analyses therefore are only suggestive, given the risk of overinterpretation. The interaction identified between ER and serum E2 in the multivariable model based on OHE and hormone factors (Table 5) supports the findings from the subgroup analyses. Further studies are needed to confirm the findings from the subgroup analyses.

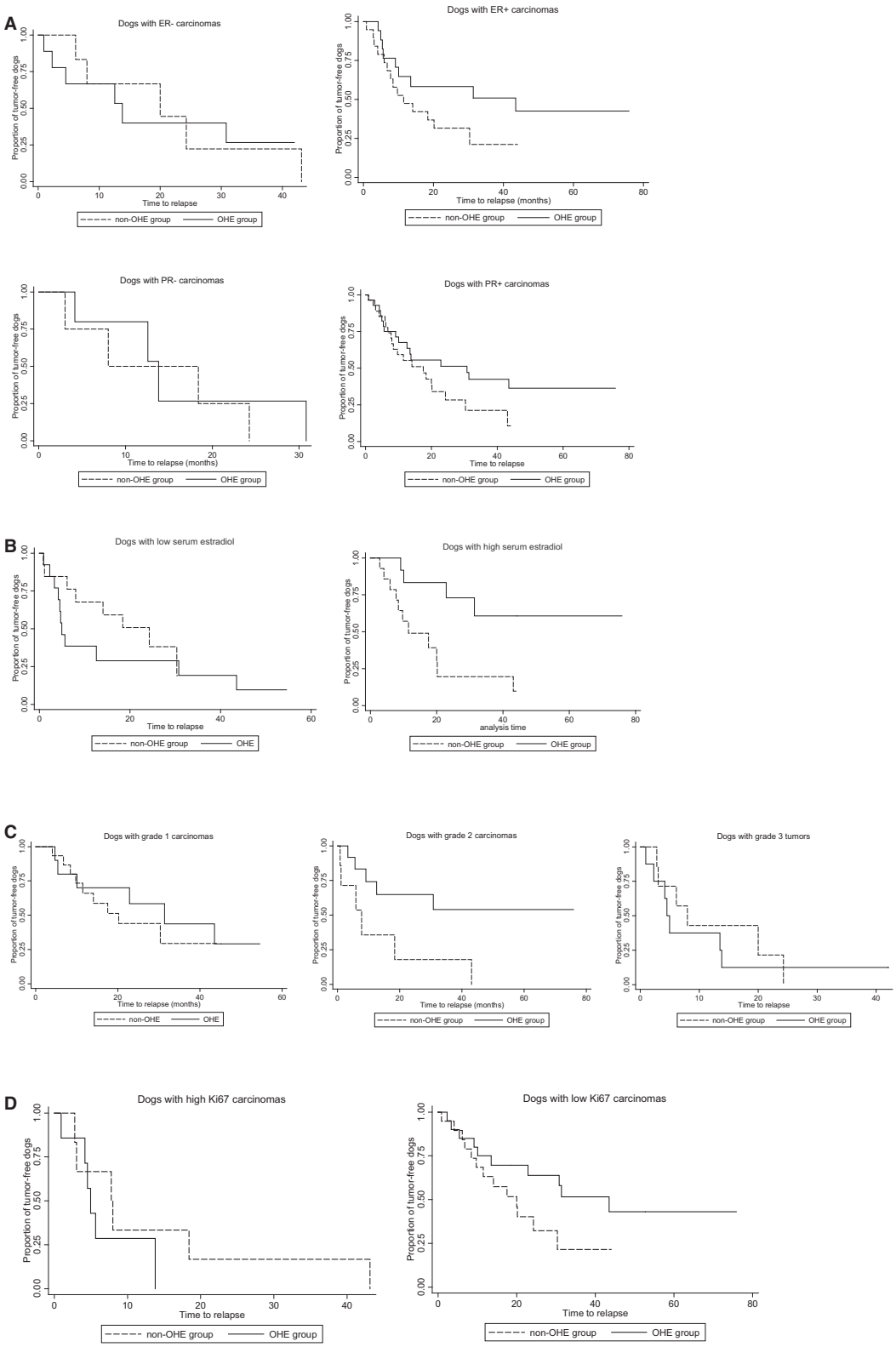

Fig 3. Effect of ovariohysterectomy (OHE) on disease-free period in selected subgroups of dogs with mammary carcinoma participating in a prospective randomized study where 31 of the dogs underwent OHE and 29 remained intact at the time of tumor surgery (solid line, OHE-group; dashed line, non-OHE group). (**A**) Effect of OHE stratified solely on tumor hormone receptor status. (**B**) Effect of OHE stratified solely on peri-surgical level of serum estradiol. (**C**) Effect of OHE stratified solely on histological grade. (**D**) Effect of OHE stratified solely on Ki67 immuno staining.

Perhaps the most intriguing finding in this study was the effect of high peri-surgical E2 on outcome, specifically the benefit of OHE. These findings are consistent with previous research documenting a significant association between a tumor's HoR status and a dog's hormonal status.[14,23,24] This effect likely is because of estrogens modulating the tumor's response to hormonal treatment by upregulating the tumor's HoRs. The positive association between E2 and tumor HoR (Table 3) supports this hypothesis. The lack of statistically significant benefit in dogs with ER/PR positive tumors may be due in part to low statistical power, but also suggests that IHC may not accurately identify which dogs that are likely to benefit from hormonal treatment. The variable E2 performed much better here, and provides a practical easy-to-perform 1-step analysis to inform treatment decisions regarding OHE in dogs with mammary tumors. Grade also was found to be associated with response to OHE. Specifically, dogs with grade 2 tumors had significantly better DFP if they were randomized to OHE compared to intact dogs. Previous studies have found an association between grade and HoR with high-grade tumors being less likely to express HoR than low-grade tumors and therefore not likely to benefit from hormonal treatment. No benefit, however, was observed in dogs with grade 1 tumors, which is not necessarily surprising. As discussed above, not all dogs require treatment beyond surgery, and dogs with grade 1 tumors fall into this category (ie, most of them are treated effectively with surgery alone). In contrast, dogs with grade 2 tumors have the potential for metastasis, but also may retain some hormonal dependence. In this subgroup additional treatment is needed, and hormonal treatment may make a difference. Our results support this conclusion, and suggest that grade can be used as a surrogate marker for hormone dependence in dogs with mammary tumors.

Our study is the first using a RCT design to determine the role of concomitant OHE in the treatment of mammary carcinoma in dogs. The effect of potential confounders was minimized by the randomization procedure, and the 2 intervention groups were equal in

Table 5. Results of the multivariable cox regression analysis of factors influencing the disease free period in 60 dogs (39 failures) with mammary carcinomas participating in a randomized trial investigating the prognostic effect of ovariohysterectomy at the time of mastectomy.

Variables	HR	SE	*P*-Value	95% CI
Models based on tumor factors which cannot be in the same model because of correlations				
Model 1[a]				
OHE[b] (yes vs no)	0.64	0.22	.194	0.33–1.26
Grade 2 (vs Grade 1)	1.22	0.51	.63	0.54–2.75
Grade 3 (vs Grade 1)	2.84	1.10	.007	1.33–6.05
Model 2[c]				
OHE[b] (yes vs no)	0.76	0.27	.44	0.38–1.52
Ki67 (high vs low)	3.46	1.31	.001	1.65–7.27
Model based on hormone factors[d]				
OHE[b] (yes vs no)	0.54	0.21	.123	0.25–1.18
E2 (high vs low)	0.16	0.13	.025	0.034–0.80
Tumor ER (pos vs neg)	0.34	0.18	.038	0.12–0.94
E2 × tumor ER	7.12	6.7	.037	1.12–44.99

[a]Number of observations was 60 dogs with 39 failures.
[b]OHE was included in the models because it was the primary investigated variable.
[c]Number of observations were reduced to 53 dogs with 35 failures because of missing values.
[d]Number of observations were reduced to 44 dogs with 29 failures because of missing values.

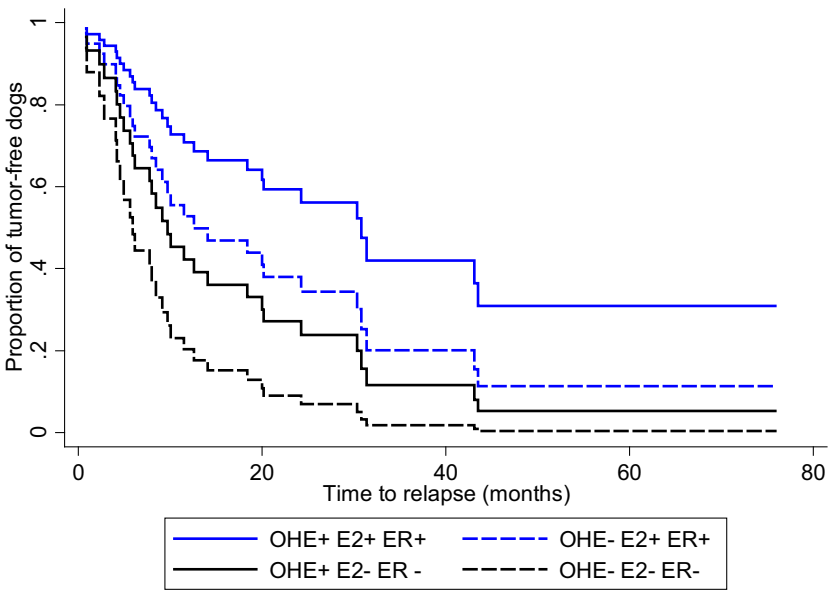

Fig 4. Disease-free period survival curves illustrating how the interaction effect found between serum estradiol (E2) and estrogen receptor (ER) status, alters the hazard of relapse in dogs with mammary carcinomas treated in a randomized clinical trial investigating the effect of ovariohysterectomy (OHE) on clinical outcome. Thirty-one dogs underwent OHE while 29 remained intact during tumor removal (n = 60, 39 failures).

almost all host- and tumor variables at the onset. Other strengths of this study were good control of anamnestic information, close clinical monitoring, long follow-up (up to 76 months) and the fact that necropsy was performed in almost all (86%) of the 50 dogs that died during the study period. However, because of the exclusion criteria, generalizability is limited to intact dogs without metastasis or previous history of malignant tumors.

In conclusion, our study indicated that CMT constitute a heterogeneous disease and that not all dogs with MC benefit from OHE at the time of tumor removal. Dogs with grade 2, ER-positive tumors, or with increased peri-surgical serum E2 represented the subset of dogs with mammary carcinomas most likely to benefit from OHE. Peri-surgical serum E2 concentration can be used as an easily available marker to identify dogs that might benefit from OHE.

Footnotes

[a] Lab Vision, Thermo Fisher Scientific, Waltham, MA
[b] MAD-004072R/D, Master Diagnostica, Granada, Spain
[c] M7047, Dako, Glostrup, Denmark
[d] EnVision developing system-HRP K4007, Dako, Glostrup, Denmark
[e] 790-2223, Ventana Medical Systems, Roche, Basel, Switzerland
[f] MAD 000310QD, Master Diagnostica, Glostrup, Denmark, Granada, Spain
[g] MAD-021881QK, UltraVision Quanto-HRP, Master Diagnostica, Granada, Spain
[h] 10-8500 mounting medium, Casa Alvarez, Madrid, Spain
[i] Stata 12, Stata Corporation, College Station, TX

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Conflict of Interest Declaration: Neither of the authors of this paper has financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of this paper.

Off-label Antimicrobial Declaration: Authors declare no off-label use of antimicrobials.

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