Prognostic significance of immunohistochemical markers and histological classification in malignant canine mammary tumours

Nieves Pastor¹ | Luis Javier Ezquerra² | Massimo Santella¹ | Nuria C. Caballé³ | Raquel Tarazona⁴ | María Esther Durán⁵

¹Veterinary Teaching Hospital, University of Extremadura, Cáceres, Spain
²Animal Medicine and Surgery Unit, Department of Animal Medicine, Faculty of Veterinary Medicine, University of Extremadura, Cáceres, Spain
³Department of Physics and Mathematics, University of Alcalá, Science Faculty, Alcalá de Henares, Spain
⁴Immunology Unit, Department of Physiology, Faculty of Veterinary Medicine, University of Extremadura, Cáceres, Spain
⁵Anatomy and Comparative Pathological Anatomy, Department of Animal Medicine, Faculty of Veterinary Medicine, University of Extremadura, Cáceres, Spain

Correspondence
Raquel Tarazona, Immunology Unit, Department of Physiology, Faculty of Veterinary Medicine, University of Extremadura, Avenida de la Universidad s/n, 10003 Cáceres, Spain.
Email: rtarazon@unex.es

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Abstract
Canine mammary carcinoma represents a model for the study of human breast cancer, although the prognostic value of various clinical, histological and immunohistochemical parameters has shown contradictory results. A prospective study, through a 4-year follow-up, was performed in 77 patients with mammary carcinoma to analyse the association between histological diagnosis, grade of malignancy, peritumoral and vascular invasion. We have also performed immunohistochemistry for the expression of oestrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) and cyclooxygenase-2 (COX-2) that define human biomarkers of disease progression and treatment response. An association between histological diagnosis and clinical stage was observed with a high proportion of complex carcinoma classified as stage I. There was a higher proportion of ER⁺/PR⁺/HER2⁻ tumours in stage I. In contrast, triple-negative tumours (ER⁻/PR⁻/HER2⁻) were found mainly in advanced clinical stages and were associated with vascular and peritumoral invasion. The tumours included in group VII (carcinosarcoma/adenosquamous carcinoma/other special types of carcinoma) had a higher expression of COX-2. The univariate analysis showed that those patients with complex carcinoma had the lowest incidence of metastases and the highest probability of survival. In contrast, a high proportion of patients with anaplastic/inflammatory carcinoma developed metastases and showed the lowest probability of survival. In addition, the estimated survival time was shorter for those patients with triple-negative tumours and those with high COX-2 expression. However, in the multivariate analysis, only the peritumoral invasion maintained its prognostic significance. In conclusion, in our study anaplastic/inflammatory carcinomas had the worst prognosis with a high proportion of triple-negative tumours in this category.

KEYWORDS
canine mammary carcinoma, histological classification, immunohistochemistry, pathology, prognosis
1 | INTRODUCTION

The prognostic evaluation of mammary cancer in veterinary medicine is based on clinical stage (tumour size, lymph node status and radiographic evidence of distant metastases), vascular invasion and clinical examination of the tumour in accordance with World Health Organization (WHO) guidelines. In 2011, Goldschmidt et al published an updated and more detailed histological classification of subtypes of canine mammary carcinomas based on the WHO criteria previously published in 1974. The prognostic significance of this classification has been analysed in several subsequent studies, and shown to be related to lymphatic invasion, distant metastases and overall survival.

Although, the only effective treatment is surgical removal of the affected glands and local lymph nodes, adjuvant therapies, such as chemotherapy or radiotherapy, are often administered in canine patients. However, there is very limited information on their efficacy. It is known that early detection is crucial for the evolution of patients with mammary tumours and that the determination of biomarkers is a key to evaluate the disease progression and response to treatments.

Canine mammary carcinoma has been shown to be a valid model for the study of breast cancer in women. For this reason, the molecular classification used in human medicine has been used to establish an immunohistochemical classification of canine mammary carcinoma. This classification includes the expression of oestrogen receptor alpha (ERα) and progesterone receptor (PR) and the over-expression of human epidermal growth factor receptor 2 (HER2) in an attempt to redefine the classification of mammary neoplasms, predict their prognosis and provide therapeutic guidelines for routine clinical practice.

Several studies have examined different diagnostic antibodies routinely used in human breast cancer to characterise molecular-based groups of canine mammary tumours, but obtained contradictory results because of the variability of the criteria used to classify breast cancer. Abadie et al, however, successfully adapted the immunohistochemical classification of human breast cancer proposed by Nielsen et al and the classification of Blows et al for canine mammary carcinoma. Despite this, immunohistochemical receptors are not routinely analysed in canine mammary tumour disease because of their high cost.

The immunohistochemical characterisation of the cyclooxygenase-2 (COX-2), an enzyme involved in the production of inflammatory mediators, has been widely studied as a prognostic factor in canine mammary carcinoma, being associated with disease progression, poor prognosis and short survival in dogs with mammary carcinomas.

Prospective studies of female dogs with mammary carcinomas are not very numerous in the veterinary literature, as well as prognostic studies with multivariate analyses. Therefore, the specific objectives of this study were to investigate the relationship between histological diagnosis and immunohistochemical classification (ER, PR, HER2 and COX-2) with clinical stage tumor/lymph node/metastasis (TNM), histological grade of malignancy, vascular invasion and peritumoral invasion, and to describe the clinical evolution of the patients (development of metastasis and cancer-specific death) based on the histological diagnosis and immunohistochemical classification.

2 | MATERIAL AND METHODS

2.1 Study sample

A prospective analysis was performed in 77 patients with malignant mammary tumours. All patients were followed up from their first visit to the Surgery Service of the Veterinary Teaching Hospital at the University of Extremadura, Spain, for assessment and treatment.

The patients included in this study were selected among 385 patients with mammary tumours that were diagnosed in the study period. The selection criteria were a diagnosis of carcinoma or carcinosarcoma from the removed tumour and the ability to carry out a long-term follow-up (January 2008-December 2012), every 6 months, or until death of the patient, excluding patients whose owners opted for adjuvant chemotherapy and patients whose owners declined regular follow-up every 6 months.

2.2 Histological study

The biopsies were sent to the Pathology Service, where they were evaluated macroscopically. The tissue was processed and embedded in paraffin blocks, sections of 5 μ were stained using the appropriate histochemical techniques.

2.3 Immunohistochemical study

The immunohistochemistry technique was performed using the EnVision FLEX Mini Kit, High pH high-sensitivity visualisation system (Dako Autostainer/Autostainer Plus, Dako). The PT Link (Dako) module was used to pretreat the samples at a maximum temperature of 95°C with the corresponding retrieval solution according to the antibody used.

The sections were incubated with the primary antibodies:

1. IO Path Mouse Anti-Human Progesterone Receptor Monoclonal Antibody. Clone PR10A9. Prediluted (Beckman Coulter).
2. Mouse Anti-Human Oestrogen Receptor α Monoclonal Antibody. Clone 1D5. Dilution 1:35 (Dako).
3. Rabbit Anti-Human HER2 Polyclonal Antibody. Dilution 1:250 (Dako).
4. Mouse Anti-Human COX-2 Monoclonal Antibody. Clone CX-294. Dilution 1:100 (Dako).

The negative control was performed with a mouse negative control (FLEX Negative Control, Mouse; Dako) and a rabbit negative control (FLEX Negative Control, Rabbit; Dako). According to the manufacturer (Dako), as positive controls, endometrium was used for
the anti-PR and anti-ER antibodies and the adrenal gland was used as the control organ for the anti-COX-2 antibody. Normal mammary glandular tissue can present low expression of HER2 antibody staining. Following American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) recommendations for humans, HER2 positivity (overexpression) was considered only for 3+ tumours.

2.4 | Statistical analysis

The epidemiological variables studied were breed, including purebred and mixed breeds, age and size including large (>50 cm) and medium to small (<50 cm). With regard to reproductive variables, data were collected on spaying prior to diagnosis and age at which it was performed, number of pregnancies, number of pseudopregnancies and hormone therapy.

Five categories were considered to assess the variable clinical stage according to the WHO's modified TNM staging system. The tumours were classified into seven categories according to their aggressiveness using the histological classification of Goldschmidt et al to obtain a significant number in each category: I-complex carcinoma, II-simple carcinoma, III-anaplastic carcinoma/inflammatory carcinoma, IV-mixed carcinoma, V-invasive micropapillary carcinoma/comedocarcinoma/solid carcinoma, VI-ductal carcinoma/intraductal papillary carcinoma and VII-carcinosarcoma/adenosquamous carcinoma/other special types of carcinoma. Grade of malignancy and the variables peritumoral invasion (defined by the presence of neoplastic cells infiltrating normal tissue adjacent to tumour) and vascular invasion were also considered. The variables development of distant metastases and cancer-specific death were defined as the period (in months) between surgical tumour removal and, respectively, the occurrence of distant metastasis or death because of the tumour.

ERx and PR immunoexpression was established according to the guidelines recommended by the ASCO/CAP adapted to the canine species. Positive immunoexpression was considered ≥2. Expression of HER2 oncoprotein was established according to ASCO/CAP recommendations for the evaluation of HER2 in humans, in which only 3+ tumours are considered positive for HER2 overexpression. Positivity for COX-2 was indicated by cytoplasmic staining. The distribution score and intensity were multiplied to obtain a total score, which ranged from 0 to 12, with scores from 0 to 5 considered low and scores from 6 to 12 considered high.

The Statistical Package for the Social Sciences version 22.0 (SPSS, Chicago, Illinois) was used for the statistical analysis. Descriptive analysis of variables, normality tests (Shapiro-Wilk and Kolmogorov-Smirnov), Pearson's chi-square test (to compare two discrete variables) and Cox regression (univariate analysis) were used. For the survival analysis, a univariate analysis was performed with censored data using the Kaplan-Meier estimator and the differences were studied with the log-rank test; with the 95% confidence interval (95% CI) that defines a range of values that contains, with at least 95% of certainty, the population mean. A Cox regression model was used to evaluate the prognostic value of the study variables.

2.5 | Cell line validation statement

Since no cell lines were used in the current study, validation testing has not been conducted.

3 | RESULTS

3.1 | Epidemiological variables

The mean age of the patients was 10 years (±2.3) (interquartile range [IQR] = 4 years), 64.9% (n = 50) were medium-small and only 35.1% (n = 27) were large. There were almost twice the number of purebred (62.3%, n = 48) than mixed-breed patients (37.7%, n = 29), with hunting breeds accounting for the largest percentage (23.4%). As regards the reproductive variables, only 11.7% (n = 9) of the patients were spayed, with the age of spaying ranging from 4 months to 4 years before surgical removal of the tumour. The mean age of spayed patients was 8 years (±2.8) (IQR = 5 years) and the mean age of unspayed patients was 9.9 (±2.4) (IQR = 4 years), without statistically significant differences. Of the patients in the sample, 74.2% (n = 57) had never been pregnant, 7.6% (n = 6) of the patients were multiparas and 18.2% (n = 14) presented one or two gestations. Information on pseudopregnancies was obtained for 81.8% (n = 63) of the patients, of which 28.6% (n = 22) had multiple pseudopregnancies. A total of 81.5% (n = 63) of the patients never received hormone therapy for oestrus suppression.

3.2 | Clinical stage

When evaluating the patients’ clinical stage prior to surgery it was observed that 50.6% (n = 39) were in stage I, 10.4% (n = 8) in stage II, 16.9% (n = 13) in stage III, 10.4% (n = 8) in stage IV and 11.7% (n = 9) in stage V.

3.3 | Histological and immunohistochemical study

The histological diagnosis of the tumours indicated that group I was the largest (25.9%, n = 20), followed by group IV and group VII. Each of these last two groups included 15.5% (n = 12) of the patients. Table S1 shows the distribution of patients in groups by histological diagnosis and epidemiological variables.

As regards the histological grade of malignancy, 31.2% (n = 24) of the patients had a low grade of malignancy, 42.9% (n = 33) had an intermediate grade and 26% (n = 20) had a high grade.

In terms of peritumoral invasion, 66.2% (n = 51) of patients with mammary carcinoma did not present invasion compared with 33.8% (n = 26) of those that did. Additionally, 71.4% (n = 55) did not present vascular invasion, whereas 28.6% (n = 22) did.

Moreover, the analysis of ER, PR and HER2 immunoexpression (Figures S1-S3) showed that 13 patients (16.9%) lacked...
immunoreactivity to these antibodies (ER+ /PR+/HER2− or triple negative), while 38 patients were classified immunohistochemically in the ER+ /PR+ /HER2− group (49.4%). Finally, 31.8% of the patients (n = 23) were classified in the ER+ /PR− /HER2− group. It should be noted that three patients belonging to the ER+ /PR− /HER2+, ER+ /PR+ /HER2− and ER+/PR−/HER2− groups, which had only one animal per group, were excluded from the statistical study (Figure 1). Of all the patients included in the study, 68.8% (n = 53) showed lack of immunoreactivity to the ER. Table S2 shows the distribution of patients according to immunohistochemical expression and their relationship with prognostic factors.

The analysis of COX-2 immunoreactivity indicated that 33.8% of the tumours presented high expression (Figure S4).

3.4 Associations between clinical and pathological variables

3.4.1 Clinical and pathological variables associated with the histological types

The variables histological diagnosis and clinical stage were significantly associated. A higher proportion of patients (46.2%, n = 18) with tumours classified as complex carcinoma were in stage I, while group III (anaplastic carcinoma/inflammatory carcinoma) had the highest percentage of patients in stage V (33.3%, n = 3) ($\chi^2; P = .005$).

As regards the histological grade of malignancy, groups III (anaplastic carcinoma/inflammatory carcinoma) and V (invasive micropapillary carcinoma/comedocarcinoma/solid carcinoma) had a significantly larger proportion of individuals with high-grade tumours than that observed in the other tumour groups ($\chi^2; P < .001$).

As for vascular and peritumoral invasion, a significantly higher proportion of patients in groups I (complex carcinoma) and IV (mixed carcinoma) did not present these types of invasion compared with the patients included in the other groups of diagnosed tumours ($\chi^2; P < .001$).

Regarding the relationship between histological diagnosis and ER, PR and HER2 expression, the proportion of ER+ /PR+ /HER2− patients in group I (complex carcinoma) was significantly higher than the proportion of patients with this immunophenotype in the other groups ($\chi^2; P < .001$). In contrast, the percentage of patients with ER+ /PR+ /HER2− expression was higher in groups IV (mixed carcinoma) and VII (carcinosarcoma/adenosquamous carcinoma/other special types of carcinoma), groups V (invasive micropapillary carcinoma/ comedocarcinoma/solid carcinoma) and VI (ductal carcinoma/intraductal papillary carcinoma) than in the other groups ($\chi^2; P < .001$).

Moreover, the proportion of triple-negative tumours (ER− /PR− /HER2−) was significantly higher in group III (anaplastic carcinoma/inflammatory carcinoma) than in the other groups studied ($\chi^2; P < .001$).

3.4.2 Clinical and pathological variables associated with ER, PR and HER2

The association between the variables ER, PR and HER2 and clinical stage was significant. There was a higher proportion of ER+ /PR+ /HER2+ tumours in stage I (73.9%, n = 17) than in the rest of the clinical stages, while triple-negative tumours corresponded to the highest stages (III, IV and V) ($\chi^2; P = .014$). Additionally, considering the histological grade of malignancy, the proportion of patients with high-grade tumours was larger (46.2%, n = 6) among those with triple-negative mammary tumours, followed by patients with ER− /PR+ /HER2− tumours (28.9%, n = 11) and the lowest proportion was detected in patients with ER− /PR− /HER2− tumours (8.7%, n = 2) ($\chi^2; P < .001$).

With regard to the relationship between the expression of these receptors and vascular and peritumoral invasion, the percentage of tumours with vascular invasion was significantly higher in the triple-negative group (46.2%, n = 6) than in the group of patients with ER+ /PR+ /HER2− tumours (28.9%, n = 11) and in tumour processes classified as ER− /PR− /HER2− tumours (13%, n = 3) ($\chi^2; P < .001$). Statistically significant differences were also observed ($\chi^2; P < .001$) when comparing the vascular invasion of mammary tumours in the ER− /PR− /HER2− group with those observed in the ER− /PR+ /HER2− group.

In the triple-negative carcinomas, evidence of peritumoral invasion was found in 53.8% (n = 7) of the tumours compared with 34.2% (n = 13) of patients belonging to the ER− /PR+ /HER2− group ($\chi^2; P < .001$) and 17.4% (n = 4) in patients with ER− /PR+ /HER2− tumours ($\chi^2; P = .02$). The proportion was also higher for the ER− /PR− /HER2− group (34.2%, n = 13) than in tumours with positive immunoreactivity for both hormone receptors (ER− /PR− /HER2−; 17.4%, n = 4) ($\chi^2; P = .02$).

3.4.3 Clinical and pathological variables associated with COX-2 expression

No statistically significant associations were found between the variables clinical stage and COX-2 enzyme expression. In contrast, when analysing the relationship between the variable histological diagnosis and COX-2 expression, the percentage of patients with complex carcinoma and a low COX-2 score was significantly higher than in the
other tumour groups. However, the patients with tumours of group VII (carcinosarcoma/adenosquamous carcinoma/other special types of carcinoma) showed a significantly higher expression of this enzyme ($\chi^2; P < .001$).

COX-2 expression was not found to have a statistically significant association with the different groups of mammary tumours according to ER, PR and HER2 expression. A trend can be observed between COX-2 expression and histological grade of malignancy. Specifically, 39.2% (n = 20) of tumours with low COX-2 expression also showed a low histological grade of malignancy, whereas only 25.5% (n = 13) of tumours with low COX-2 expression showed a high histological grade of malignancy ($\chi^2; P = .07$).

Moreover, no statistically significant relationship was found between the expression of this enzyme and vascular and peritumoral invasion.

### 3.5 | Univariate survival analysis

Of the patients studied, 41.6% (n = 32) developed distant metastases during follow-up (95% CI: 29.9, 53.2), of which 56.3% (n = 18) presented distant metastasis at 12 months and 43.7% (n = 14) developed them during the second year, compared with 58.4% (n = 45; 95% CI: 46.8, 70.1) who showed a favourable evolution in relation to this variable. Median time to distant metastasis was 11.1 (±2.2) months (95% CI: 6.9 and 15.6), being the lungs (80%, n = 25), lymph nodes (16%, n = 5) and central nervous system (4%, n = 2) the distant metastasis sites, with a one-year and two-year probabilities of distant metastasis of 0.7 and 0.8, respectively.

Otherwise, median cancer-specific death was 40.9 (±3.7) months (95% CI: 33.7 and 48.1), with a probability of cancer related death at 1-year and 2-year postmastectomy of 0.3 and 0.4, respectively. In our study, 46.8% (n = 36) of the patients died during the study follow-up period. Most cancer related deaths were observed during the first year after diagnosis. Thus, 26 of 36 patients (72.2%) died during the first year and 10 patients (27.8%) died in the second year of follow-up.

### 3.5.1 | Prognostic significance of the histological classification

Patients with complex carcinoma were found to present the lowest incidence of distant metastasis (15%, n = 3). In contrast, patients of group III (anaplastic carcinoma/inflammatory carcinoma) developed the highest number of distant metastases (85.7%, n = 6), with a statistically significant difference ($\chi^2; P = .028$) between the two groups.

The survival analysis for the groups according to their histological diagnosis showed that the patients with the highest probability of survival were those diagnosed with complex carcinoma (group I) with an estimated survival time of 62.7 months (95% CI: 52.9 and 72.5), followed by patients with group VI carcinomas (ductal carcinoma/ intraductal papillary carcinoma) with 46.4 months (95% CI: 26.9 and 65.8). In contrast, patients with group III tumours (anaplastic carcinoma/inflammatory carcinoma), showed the lowest estimated survival probability with 5.7 months (95% CI: 0.0 and 12.2) (Table 1). The survival rate of each group using the Kaplan-Meier curve is shown in Figure 2.

Univariate analysis using chi-square test showed statistically significant differences ($P < .005$) in survival according to histological subtype (Table 2). However, in the Cox regression no statistically significant relationship was observed between histological subtype and survival ($P = .06$) or incidence of metastasis ($P = .06$).

### 3.5.2 | Prognostic significance of the immunohistochemical classification

Considering the variable metastasis development, it is observed that there was no statistically significant relationship between the proportion of patients who developed distant metastasis and the three immunohistochemical expression groups ($\chi^2; P = .7$). Likewise, there was not statistically significant relation when the Cox regression model was used ($P = .2$).

The survival analysis for the three groups of patients classified according to ER, PR and HER2 showed that the group with double positivity for both hormone receptors (ER$^+$/PR$^+$/HER2$^-$) had the highest probability of survival, with an estimated survival time of 55.1 months (95% CI: 43.5 and 66.7). The patients in the group that expresses only the progesterone receptor (ER$^-/PR^+$/HER2$^-$) had an estimated survival time of 34.1 months (95% CI: 25.8 and 42.3) and a median survival of 32 months (95% CI:19.3 and 44.7), whereas the group lacking expression for the three receptors (triple negative) had the lowest probability of survival, with an estimated survival time of 27.6 months (95% CI: 15.4 and 39.7) and a median survival time of 34 (95% CI:0.8 and 67.2). The Kaplan-Meier survival curve clearly shows the trend for the three groups assessed (Figure 3). The survival analysis performed either by chi-square test (Table 2) or Cox regression showed no significant results ($P = .7$).

### 3.5.3 | Prognostic significance of the COX-2 enzyme

No association was found when comparing COX-2 expression with the development of distant metastases ($\chi^2; P = .283$). However, a Kaplan-Meier survival analysis showed that patients presenting tumours with high COX-2 expression were less likely to survive than those with low expression. Specifically, the mean estimated survival time for patients with high expression was 26.4 months (95% CI: 17.4 and 35.3) and presented a median survival time of 20 (95% CI:0 and 42.9), while it was 46.2 months for patients with low expression of COX-2 (95% CI: 37.6 and 54.8). The Kaplan-Meier survival curve shows this trend (Figure 4).

Univariate analysis using chi-square test showed statistically significant differences ($P = .02$) in survival according to COX-2 expression (Table 2). The analysis using the Cox regression model did not
show a statistically significant relationship between COX-2 expression and the appearance of distant metastases ($P = .1$) or patient survival ($P = .3$).

### 3.6 Multivariate survival analysis

A multivariate analysis was performed to assess the joint effect of histological diagnosis, ER, PR and HER2 expression, histological grade of malignancy, peritumoral and vascular invasion and COX-2 enzyme (independent variables) on the follow-up variable death (dependent variable) (Table 2). Only the variable peritumoral invasion ($P < .001$) remained as an independent prognostic factor for death in the final model.

### 4 Discussion

Canine mammary tumours occur in elderly females, usually between 8 and 10 years of age and may vary according to the natural life span of the breed. This is especially significant in Europe, where females are not spayed at an early age, which coincide with our results.

As regards the size of the patients, our findings are in line with other epidemiological studies where small and miniature breeds are over-represented. However, Itoh et al indicated that small breeds are the least predisposed to mammary carcinoma. In general, our study shows a greater prevalence of pure-bred individuals vs mixed breeds, as reported by other authors, although the presentation will vary depending on the geographical area being analysed.

In relation to the histological diagnosis, the most commonly diagnosed tumour types are complex carcinoma, followed by mixed carcinoma and carcinosarcoma/adenosquamous carcinoma/other special types of carcinoma, as also reported by other authors. Our data confirm those of other authors, who have found a statistically significant association between a better evolution for complex carcinomas and mixed carcinomas and a worse prognosis for other types of mammary tumours such as inflammatory carcinomas, anaplastic carcinomas, carcinosarcomas, comedocarcinomas, adenosquamous carcinomas and simple carcinomas, with high rates of local recurrence and metastases. Despite the strong association between the histological classification of canine mammary tumours and survival, the multivariate analysis showed that it is not significant, confirming that it represents a weak prognostic factor, rarely retained in multivariate survival analyses in breast cancer.

Moreover, in line with other studies, we observed a strong relationship between histological diagnosis and other clinicopathological parameters with prognostic value, as clinical stage, grade of malignancy, and presence of vascular and peritumoral invasion. Unaltered canine mammary tissue and benign neoplastic processes express both ER and PR, while low ERx expression has been associated with malignant neoplastic mammary processes with worse prognosis for the patient. It has been shown that primary and
metastatic malignant canine mammary tumours frequently express lower levels of ER and PR compared with benign tumours or healthy mammary tissues, suggesting that canine mammary tumours lacking ER and PR expression are more likely to progress than ER+ and PR+ tumours. This is consistent with our results, as patients with triple-negative tumours were found to have a lower survival rate.

In our work we have verified that tumours with an ER+/PR+/HER2− profile present low malignancy and absence of vascular and peritumoral invasion, whereas triple-negative tumours (ER−/PR−/HER2−) show high malignancy and both types of invasions. Im et al. reported the associations between luminal A breast carcinomas (ER+ or PR+ and HER2−) and lower histological grade and the absence of lymphatic invasion and then between triple-negative tumours with higher histological grade and the presence of vascular and peritumoral invasion, coinciding with those described for breast cancer in human medicine.

Several studies highlight that the proportion of tumours with PR expression is higher (72%-76%) than the proportion of tumours with ER expression (30%-50%). In our study, 76.6% of tumours expressed PR, but PR expression was not associated with increased survival for these patients. These studies reviewed reported that ER expression is associated with good-prognosis features such as lower stage, whereas PR expression was significantly associated with longer postoperative survival. Our study corroborates these findings, as ER+/PR+/HER2− expression was significantly associated with stage I and better prognosis in the patients' evolution.

In our study, although there are no statistically significant differences according to their immunohistochemical profile, the patients of the ER+/PR+/HER2− group have a greater survival probability in a 4-year follow-up as opposed to the triple-negative patients. It should be noted that, in our sample, HER2 oncprotein overexpression was observed in two tumours. For this reason, no significant differences in survival were found, as described by other authors, suggesting the
questionable value of the role of HER2 oncprotein in mammary carcinomas in the canine species.\textsuperscript{44}

Several studies on COX-2 expression have shown that immunoreactivity is more frequent and more intense in malignant mammary tumours than in benign ones, as occurs in breast cancer in women, the percentages of COX-2 expression vary from 56\%\textsuperscript{45} to 100\% of malignant mammary tumours in the canine species.\textsuperscript{17,46,47} Our results differ from these findings, since almost half of the tumours analysed lack immunostaining for the COX-2 enzyme, probably because of the use of different antibodies or different methods of quantifying immunorepression. Likewise, a statistical relationship between COX-2 expression and clinical stage has not been established.

In our work we have observed a relationship between more aggressive histological types of tumours and high COX-2 expression,\textsuperscript{17,47} as we verified that tumours classified as group VII show the highest immunorepression. Moreover, in our study no statistically significant relationship between the expression of this enzyme and clinical stage, vascular and peritumoral invasion or the development of distant metastases was found, as reported Hoellen et al in human breast cancer.\textsuperscript{48} However, tumours with a low histological grade of malignancy tend to have a low COX-2 expression. Anadol et al proved the existence of a statistically significant relationship between COX-2 mRNA levels and the histopathological grade and correlated these results with a greater aggressiveness of the tumour,\textsuperscript{49} as established for breast cancer in women.\textsuperscript{49} Our work supports this evidence, since it is observed that patients presenting tumours with a high expression of the COX-2 enzyme are less likely to survive than those with low expression. However, this parameter loses its prognostic value in the multivariate analysis, as reported previously by other authors.\textsuperscript{18}

In conclusion, patients with tumours classified as group III (anaplastic carcinoma/inflammatory carcinoma) tend to be in stage V of the disease, having a high histological grade of malignancy, showing both vascular and peritumoral invasion, and are associated with a poor prognosis.

As regards ER, PR and HER2 immunoreexpression, tumours without ER, PR and HER2 (triple negative) are associated with stage III, IV and V of disease, a high histological grade of malignancy, the presence of vascular and peritumoral invasion and a poor prognosis; with ER positive tumours being the ones with the best prognosis.

As for the COX-2 enzyme expression, high expression for this enzyme was associated with carcinomas included in group VII (carcinoma/adenosquamous carcinoma/other special types of carcinoma) and a poor prognosis.

Finally, in the multivariable model only peritumoral invasion was found to be an independent prognostic factor.

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CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

AUTHOR CONTRIBUTIONS

Nieves Pastor, María Esther Durán and Raquel Tarazona designed the study. María Esther Durán performed laboratorial techniques and pathological diagnosis. Nieves Pastor, Luis Javier Ezquerra and Massimo Santella accomplished surgical treatment and clinical follow-up. Nuria C. Caballé and Nieves Pastor analysed the data and generated figures and tables. Luis Javier Ezquerra and Massimo Santella assisted in the interpretation of data.

Nieves Pastor, María Esther Durán and Raquel Tarazona participated in writing and final editing of the manuscript. All authors have read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Raquel Tarazona https://orcid.org/0000-0002-2926-6377

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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