Paper

Adjuvant therapy for highly malignant canine mammary tumours: Cox-2 inhibitor versus chemotherapy: a case–control prospective study

C. Arenas, L. Peña, J. L. Granados-Soler, M. D. Pérez-Alenza

Cyclooxygenase-2 (Cox-2) enzyme participates in different steps of the carcinogenetic process and in canine mammary tumours (CMTs), a high expression of Cox-2 is associated with malignancy and tumour angiogenesis. The objectives of the study were to evaluate the disease-free survival (DFS) and overall survival (OS) of a Cox-2 inhibitor as adjuvant therapy in dogs with highly malignant (HM)-CMTs and compare it with that of dogs treated with chemotherapy and with control dogs. Twenty-eight dogs were prospectively included. After surgery, dogs were alternatively allocated into two treatment groups (chemotherapy with mitoxantrone n=8; Cox-2 inhibitor, firocoxib n=7). Control group (n=13) included dogs whose owners rejected adjuvant therapy. All dogs were followed up for two years or until death. The DFS was significantly higher in dogs that received adjuvant treatment (mitoxantrone or firocoxib) (P=0.030) than in control dogs. Dogs on firocoxib treatment had significantly higher DFS (P=0.015) and OS (P=0.048) than control dogs. The DFS and OS of dogs on mitoxantrone treatment were not statistically different from controls. In conclusion, this study supports the use of firocoxib for the treatment of HM-CMTs. Further studies are needed to compare the efficacy of chemotherapy drugs versus Cox-2 inhibitors as adjuvant treatment in these cases.

Introduction

Canine mammary tumours (CMTs) are the most common neoplasms in female dogs. Approximately 50 per cent of CMTs are malignant, many of which metastasise (Perez Alenza and others 2000).

Cyclooxygenase (Cox) enzymes catalyse prostaglandin formation from arachidonic acid. There are two Cox isoforms. Cyclooxygenase-1 (Cox-1) is constitutively expressed in many tissues, while cyclooxygenase-2 (Cox-2) is inducible by growth factors, inflammatory stimuli and several oncogenes (Fosslien 2000; Yoshimura and others 2003). Cox-2 participates in different steps of the carcinogenetic process, including stimulation of tumour angiogenesis, decreases tumour apoptosis, increases invasion and metastasis and tumour-associated inflammation (Hayes 2007; Tsujii and others 1998).

Angiogenesis, the recruitment of new blood vessels, plays a crucial role in tumour growth, and it is an essential component of the metastatic pathway, as it enhances entry of tumour cells into the circulation (Hanahan and Folkman 1996; Zetter 1998).

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A number of studies have demonstrated that the vascular density of a tumour is correlated with metastasis (Zetter 1998) and some studies have shown that treating experimental animals with primary tumours with angiogenesis inhibitors decreases the formation of metastatic colonies (Taylor and Folkman 1982).

Several immunohistochemical studies have shown that Cox-1 and Cox-2 are expressed in CMTs and also that a high expression of Cox-2 is associated with malignancy, it is related to a poor prognosis and it is also associated with tumour angiogenesis in CMTs (Dore and others 2003; Queiroga and others 2007). These findings suggest that Cox-2 inhibitors might be useful in the treatment of malignant CMTs.

There are also some studies which have evaluated the efficacy of several postsurgical adjuvant chemotherapy protocols in the treatment of malignant CMTs (Karayannopoulou and others 2001; Simon and others 2006; Tran and others 2014). One study carried out by Lavalle and others (2012) found that several adjuvant protocols (including chemotherapy alone or in combination with NSAIDs) for advanced CMTs led to a statistically significant longer overall survival (OS) when compared with surgical treatment alone.

The aims of this study were to evaluate the efficacy (disease-free survival (DFS) and OS) and toxicity of a Cox-2 inhibitor (firocoxib) as adjuvant therapy after surgery for highly malignant (HM)-CMTs and compare it with that of dogs treated with surgery alone or with surgery and chemotherapy.

Materials and methods

Animals

Dogs diagnosed with HM-CMTs at the Veterinary Teaching Hospital (University Complutense, Madrid) (VTH-UCM) were

prospectively enrolled from September 2008 to June 2011. Inclusion criteria included the presence of at least one HM-CMT defined as a histological malignant grade III and/or a clinical stage IV (Lana and others 2007).

Clinical history and reproductive status were obtained. All patients were physically examined and clinical stage was determined using the modified World Health Organization (WHO) staging system (Lana and others 2007). Complete blood count (CBC) and serum biochemistry were performed. Thoracic radiographs were performed to investigate the presence of metastases. Surgical resection of all mammary nodules and the lymph nodes involved was performed as described (Lana and others 2007) and subsequent histological evaluation of surgical specimens was performed. All the animals included in the study had clean margins confirmed by the pathologist. Owners were informed about the prognosis, and adjuvant therapy was recommended for all of the dogs. Dogs whose owners accepted adjuvant therapy were treated with either mitoxantrone or firocoxib. In order to eliminate bias, treatment assignment was performed by a nurse not involved in the study. This was accomplished by using a table which alternatively (1:1) allocated the dogs into the two treatment groups (mitoxantrone or firocoxib). Dogs whose owners rejected adjuvant therapy were included as controls. All dogs entering the study had normal kidney and hepatic function based on biochemistry. Dogs in group A (n=8) received five doses of mitoxantrone every 21 days (5.5 mg/m^2 intravenously); dogs in group B (n=7) received firocoxib[i] (5 mg/kg/day orally) during 24 months. Group C (n=13) comprised dogs for which owners had declined adjuvant therapy (control group). Dogs in group A were reviewed every 21 days during the first four months and every three months afterwards. Re-evaluations consisted of physical examination and evaluation of the mammary region and regional lymph nodes. A CBC was performed before the administration of each chemotherapy cycle. CBC and serum biochemistry (urea, creatinine, electrolytes, alanine aminotransferase (ALT) and alkaline phosphatase (ALP)) were performed every six months afterwards. Toxicity was graded with the Veterinary Cooperative Oncology Group Common Terminology Criteria for Adverse Effects (2011). Dogs in group B were reviewed one month after diagnosis and every three months thereafter. Serum biochemistry was performed every three months for the first nine months and every six months afterwards. Dogs in group C were reviewed one month after diagnosis and every three months thereafter. Bloods were not taken for dogs in group C during follow-up. Thoracic radiographs were taken every three months in all of the cases (groups A, B and C). All dogs were followed up for a period of two years or until death. Date and cause of death were recorded. Written informed consent was obtained from owners for inclusion of dogs in the study. The study protocol was reviewed and approved by the VTH-UCM Ethics Committee.

Histopathology and immunohistochemical examination

For histopathology and immunohistochemistry studies, samples were fixed in formalin, routinely processed and diagnosed following the WHO classification system of CMTs (Goldschmidt and others 2011). In dogs with more than one malignant CMT, the neoplasia with the more aggressive histological features was chosen in order to perform the immunohistochemical study and the statistical associations with the follow-up variables.

Immunostaining was performed with an automatic immunostainer device[ii] on deparaffinised sections after a high-temperature antigen retrieval protocol on EDTA buffer (pH 8.0) in a pre-treatment (PT) module device[iii] for 20 minutes at 95°C, cooled down to 60°C and rinsed in warm tap water. The primary antibodies used were prediluted monoclonal mouse anti-human Ki-67[iv] incubated one hour at room temperature and a prediluted rabbit monoclonal anti-human Cox-2[v] (clone SP21, with known reactivity for canine tissues, Dias Pereira and others 2009) incubated 2.5 hours. A commercial detection system kit was used[vi]. The slides were counterstained in haematoxylin, washed, dehydrated, cleared in xylene and mounted with dibutyl phthalate xylene.

Ki-67 immunoexpression was evaluated in all of the malignant tumours. For quantification of immunolabelling, the proportion of positive neoplastic cells in each sample was calculated (Peña and others 1998) and the cell proliferation index (Ki-67) was determined with a computer-assisted image analyser[vi] as the percentage of positive brown nuclei in the tumour cells in 10 representative fields.

Cox-2 immunoexpression was evaluated in all of the malignant tumours. For this purpose, an immunohistochemical score (IHS) (grades 0–3) previously described (Clemente and others 2013) was used, based on the percentage of immunostained cells and the labelling intensity of immunoreactive cells.

Statistical analysis

The results were analysed using a statistical software package[vii]. Descriptive statistics were performed for each group, including epidemiological, clinical, histopathological and follow-up variables. Epidemiological and clinical variables included were age, breed, reproductive status, previous oestrus, preventive hormonal treatments, regularity of oestrus, number of malignant tumours/animal and tumour size. For statistical purposes, tumour size was analysed using a previous reported classification (Peña and others 2013). Additional categorical variables included skin ulceration, skin adherence, adherence to underlying tissues, lymph node metastasis, distant metastasis, modified WHO clinical stage and type of surgery. Histological variables evaluated were as follows: histological diagnosis, histological malignancy grade (HMG), Ki-67 cell proliferation index and Cox-2 IHS. In animals with more than one malignant tumour, the most malignant tumour was selected for statistical purposes. Due to the large number of histological tumour types, in order to perform statistical analyses, tumours were grouped based on biological behaviour and morphology into three categories (histological variable named HD3) (Peña and others 2013) as follows: group 1, which included in situ carcinoma, simple carcinoma, carcinoma arising in a mixed tumour, complex-type carcinoma, mixed-type carcinoma, ductal carcinoma and adenosquamous carcinoma. Group 2, which included solid carcinoma, comedocarcinoma, carcinoma and malignant myoepithelioma and anaplastic carcinoma. Group 3 included other histological types (eg lipid-rich carcinoma, malignant myoepithelioma). Local recurrence, distant metastases and death were considered as follow-up variables for the three groups. In groups A and B, toxicity was also evaluated.

Kaplan–Meier survival curves were constructed for the DFS and OS. OS was chosen instead of disease-specific survival to avoid under-reporting of disease-specific deaths. DFS was calculated as months from surgery to tumour recurrence. Dogs were censored at death without disease or without tumour recurrence. OS was calculated as time (months) from surgery to death attributable to any cause or to the end of study period (24 months). Dogs were censored if they were alive at the end of the study. A multivariate analysis (Cox regression model) was applied to evaluate the influence of clinical, histological and immunohistochemical variables previously described on

[i]Previcox, Merial Laboratorios, Spain, SA
[ii]Lab Vision Autostainer, Thermo Scientific, Thermo Fisher Scientific, USA
[iii]Lab Vision PT module, Thermo Scientific, Thermo Fisher Scientific, USA
[iv]Clone SP6. Master Diagnóstica SL, Granada, Spain
[v]Clone SP21. Master Diagnóstica SL, Granada, Spain
[vi]MAD-021881QK, UltraVision Quanto-HRP; Master Diagnostica, Granada, Spain
[vii]SPSS V.19.0. SPSS, Chicago, Illinois, USA

dependent follow-up variables (DFS and OS). For all the statistical analyses, a $P \leq 0.05$ was considered significant.

Results

Animals

Twenty-eight female dogs were included in the study. A detailed description of each animal included in the study is listed in Tables 1–3. The mean age (±sem) of the dogs was 10.8 ±2.0 years (range 6–14). Twenty-one dogs (75 per cent) were entire females and the remaining seven (25 per cent) were neutered. Three dogs (10.7 per cent) had received hormonal treatments to prevent oestrus and six dogs (21.4 per cent) had presented irregular oestrous cycles.

Characteristics of the tumours

Two dogs (7.1 per cent) had a small-sized tumour (<1 cm), nine (32.1 per cent) had an intermediate tumour/s (1–2.9 cm) and seven dogs (60.7 per cent) had a large-sized tumour/s (>3 cm). Two dogs (7.1 per cent) presented tumour ulceration, in 10 dogs (35.7 per cent) the tumour was fixed to the skin and in 5 dogs (17.9 per cent) the tumour was fixed to the underlying tissues. Regional mastectomy was performed in 11 dogs (39.3 per cent), whereas radical mastectomy was done in the remaining 17 cases (60.7 per cent). All entire bitches were ovariohysterectomised at the time of surgery.

Histological diagnosis of the tumours

Fourteen dogs (50 per cent) presented one malignant mammary tumour, seven dogs (25 per cent) presented two malignant mammary tumours and three dogs (25 per cent) presented three malignant mammary tumours. Seven (25 per cent) dogs had solid carcinoma, five dogs (17.9 per cent) had comedocarcinoma, three dogs (10.7 per cent) had adenosquamous carcinoma, two dogs (7.1 per cent) had carcinoma and malignant myoepithelioma, two dogs (7.1 per cent) had tubulopapillary carcinoma, two dogs (7.1 per cent) had lipid-rich carcinoma and one (3.6 per cent) each of the following tumours: carcinosarcoma, anaplastic carcinoma, complex-type carcinoma, tubular carcinoma, ductal carcinoma and fibrosarcoma. Six tumours (21.4 per cent) were grouped on HD3 group 1, 15 tumours (53.6 per cent) on group 2 and 7 tumours (25 per cent) on group 3. All tumours were graded as HMG III.

Clinical stage

At diagnosis, six dogs (21.4 per cent) presented inguinal lymph node metastasis and one (3.6 per cent) axillary lymph node metastases. None of the dogs presented distant metastasis at diagnosis. Nine dogs (32.1 per cent) were classified as clinical stage I, 10 dogs (33.7 per cent) as clinical stage II, 2 dogs (7.1 per cent) as clinical stage III and 7 dogs (25 per cent) as clinical stage IV.

The epidemiological, clinical or histological variables (including clinical stage and HD3) between the dogs from the three groups were not statistically different ($P > 0.05$).

Ki-67 and Cox-2 immunohistochemical expression

Ki-67 immunostaining was nuclear and varied in intensity in all the tumours evaluated (n=28). Mitotic cells exhibited chromosomal Ki-67 reactivity. The Ki-67 index ranged between 18 per cent and 72 per cent. In group A, mean (±sem) Ki-67 index was 44±19.4; for dogs in group B, mean Ki-67 index was 32±8.4 and for dogs in group C, mean Ki-67 index was 45±12.7. The difference in Ki-67 immunoexpression between groups was not statistically significant (P=0.243).

Cox-2 expression was present in all of the tumours. One tumour was classified as low positive (group C), 6 were classified as moderate positive (3 from group A, 2 from group B and 1 from group C) and 21 were classified as intense positive (5 from group A, 5 from group B and 11 from group C). The difference in Cox-2 expression between groups was not statistically significant (P=0.845). The cells that showed the highest cytoplasmic immunoexpression were as follows: (a) HM isolated neoplastic cells infiltrating the mammary gland stroma; (b) neoplastic cells surrounding necrotic areas and (c) neoplastic cells in emboli within the lumen of lymphatic vessels.

Adjuvant therapy and toxicity

In group A, 7/8 dogs received five cycles of mitoxantrone. One dog developed neutropenia (grade 3) and gastrointestinal toxicity (anorexia and diarrhoea) (grade 2) and mitoxantrone was stopped after the first cycle. The dog recovered after receiving symptomatic therapy. Other side effects observed in this group were diarrhoea and anorexia (grade 1, n=1), vomiting and anorexia (grade 1, n=1) and neutropenia (grade 1, n=1; grade 2, n=3) (n=4). Dogs in group B received oral firocoxib during 11–24 months. Anorexia (grade 1) was observed in two dogs. In two cases. urea (grade 2, n=2) and creatinine (grade 1, n=1; grade 2, n=1) increased during the study period and the dog was started on a renal diet. In both cases, urea and creatinine values were within the normal range at the end of the study period. Creatinine levels did not increase above the upper reference range in any of the cases. Liver enzymes did not significantly change during the follow-up period in the dogs (groups A and B).

Follow-up

In group A, three dogs (37.5 per cent) developed local recurrence and two dogs (25 per cent) developed pulmonary metastasis. At the end of the study (24 months), 3/8 (37.5 per cent) dogs were still alive. One of these dogs had evidence of local recurrence. In four dogs, the cause of death was related to the tumour and one dog died due to congestive heart failure.

In group B, two dogs (28.6 per cent) developed local recurrence and were still alive at the time of censorship and one dog (14.3 per cent) developed pulmonary metastasis. At the end of the study period, 4/7 (57.1 per cent) dogs were still alive. In one dog, the cause of death was related to the tumour, one dog died due to signs related to advanced age and one due to a primary intestinal neoplasia.

In group C, one dog (7.7 per cent) developed local recurrence (and was still alive at the time of censorship) and nine dogs (69.2 per cent) developed pulmonary metastasis. All dogs that developed distant metastasis were euthanased because of disease progression. At the end of the study, 2/13 (15.4 per cent) dogs were still alive. One dog died due to diabetes mellitus and another was euthanased due to signs related to age.

Survival analysis—DFS

The DFS of dogs in group A was 14.3±8.6 months (median 10 ±7.9), 21.6±6 months (median 24±4.4) for dogs in group B and 10.1±5.8 months (median 7±1.3) for dogs in group C (Fig 1). Treatment was significantly associated with DFS (P=0.030). The DFS of dogs treated with mitoxantrone was not significantly different from dogs treated with firocoxib. The DFS of dogs on mitoxantrone treatment was not significantly different (P=0.31) from the DFS of control dogs. However, the DFS of dogs on firocoxib treatment was significantly higher (P=0.015) compared with control dogs. The DFS of dogs with clinical stage IV was shorter (mean 9.3±1.6; median 7±1.3 months) compared with (mean 15.9±1.9; median 20±4.7 months) that observed in dogs with clinical stages I, II and III (P=0.06).

Survival analysis—OS

The OS (mean±sem) for dogs in group A (mitoxantrone) was 16.5±2.6 months (median 18±8.5 months), for dogs in group B (firocoxib) was 19.4±2.1 months (median could not be calculated for this group as four dogs were still alive at the time of censorship) and for dogs in group C (control) was 12.7 ±1.8 months (median 11±2.4 months) (Fig 2). The one-year survival fractions were 62 per cent, 86 per cent and 47 per cent for dogs in groups A, B and C, respectively. The OS curves were statistically compared using log-rank Cox-Mantel analysis. The OS of dogs treated with mitoxantrone was not significantly different (P=0.21) from the OS of control dogs. However, the OS of

TABLE 1: Epidemiology, clinical and tumour characteristics of dogs included in group A (mitoxantrone)

Breed	Age (years)	Neutering status	Hormonal treatments	Regularity of oestrus	Number of malignant tumours	Tumour size (cm)	Fixation to muscles	Tumour ulceration	Clinical stage	Surgery	Histological diagnosis	Cox
Toy Poodle	11	E	N	R	1	2	N	N	IV	Reg	Tubular carcinoma	3
Cocker Spaniel	6	E	N	IR	1	6	N	N	III	Reg	Ductal carcinoma	2
Golden Retriever	13	S	Y	R	2	3	N	Y	IV	Reg	Comedocarcinoma	3
Cocker spaniel	10	E	N	R	1	8	Y	N	IV	Reg	Adenosquamous carcinoma	3
Labrador retriever	10	S	N	R	1	3	N	N	IV	Rad	Tubulopapillar carcinoma	3
West Highland	10	E	N	IR	3	1.5	Y	N	I	Reg	Solid carcinoma	2
Pitbull Terrier	9	E	N	R	2	5	N	N	II	Rad	Fibrosarcoma	3
Toy Poodle	12	E	N	R	1	2	N	N	I	Rad	Comedocarcinoma	2

E, entire; IR, irregular; N, no; R, regular; Rad, radical mastectomy; Reg, regional mastectomy; S, spayed; Y, yes

dogs on firocoxib treatment was significantly higher (P=0.048) compared with control dogs.

Multivariate analysis

To determine the independent prognostic value of clinical and histopathological variables in a combined model of possible predictor variables, Cox regressions of DFS and OS were performed. Lymph node involvement was associated with a reduced DFS (P=0.027, HR 0.3, CI 95 per cent 0.096–0.867) and treatment was associated with a prolonged DFS (P=0.014, HR 0.4, CI 95 per cent 0.202–0.867). Treatment was selected as an independent factor affecting OS (P=0.044, HR 0.5, CI 95 per cent 0.262–0.982).

Discussion

In dogs with HM-CMTs, the probability of tumour recurrence after surgery and death due to the disease is elevated (Lana and others 2007). Surgical excision remains the treatment of choice, but adjuvant therapy is recommended (Sorenmo 2003; Karayannopoulou and others 2005; Peña and others 2013). However, there is controversy regarding the real benefit of some adjuvant treatments in dogs with HM-CMTs (Simon and others 2006; Marconato and others 2008). From the results of authors' investigation, Cox-2 inhibitors seem to be safe and might have anti-tumoural effect in HM-CMTs, possibly increasing the OS and DFS of affected dogs when compared with surgery alone.

It has been reported that tumour size, lymph node metastasis and clinical stage are clinical factors related to prognosis (Perez Alenza and others 2000; Karayannopoulou and others 2005; Lavalle and others 2012). Animals included in this study were similar in all clinical and pathological characteristics except in clinical stage and lymph node involvement at diagnosis, and this latest variable was selected as an independent factor related to DFS. Even though the distribution of clinical stages was not statistically different between the three groups, four out of eight dogs included in group A were clinical stage IV, which might be a source of bias for the present study. Other clinical factors such as number of malignant tumours, tumour ulceration and fixation to muscles and skin were not correlated with a poor prognosis; however, these results must be interpreted with caution as to the population selected was very similar and sample size was reduced.

In the present stydy, only HMG III tumors were included, and Ki-67 was found to be positive in all of them, in accordance with others (Morris and others 2009; Clemente and others 2013), with elevated mean vales of Ki-67 in all of them. Ki-67 labelling in the CMTs studied was localised in the nuclei and showed some variability in intensity both within and among specimens, as previously observed (Geraldes and others 2000; Morris and others 2009). This study confirms that Ki-67 proliferation index is associated with a high grade of malignancy and poor prognosis.

Cox-2 expression was detected in all the tumours, the majority (75 per cent) being classified as IHS grade 3, which supports previous findings documenting that a high expression of Cox-2 is associated with malignancy and with histological tumour type

TABLE 2: Epidemiology, clinical and tumour characteristics of dogs included in group B (firocoxib)

Breed	Age (years)	Neutering status	Hormonal treatments	Regularity of oestrus	Number of malignant tumours	Tumour size (cm)	Fixation to muscles	Tumour ulceration	Clinical stage	Surgery	Histological diagnosis	Cox
Cocker Spaniel	14	E	N	R	1	0.5	N	N	I	Reg	Comedocarcinoma	2
Cocker Spaniel	13	E	N	R	3	3	N	N	II	Rad	Solid carcinoma	2
Mixed	14	E	N	IR	1	3	N	N	II	Reg	Lipid-rich carcinoma	3
Mixed	10	S	N	R	3	5	Y	N	IV	Rad	Carcinoma sarcoma	3
Cocker Spaniel	13	S	N	R	3	0.8	N	N	I	Rad	Carcinoma and malignant myoepithelioma	3
Cocker Spaniel	13	E	N	R	3	4	N	Y	II	Reg	Solid carcinoma	3
Yorkshire Terrier	9	E	N	R	2	3	N	N	II	Reg	Adenosquamous carcinoma	3

E, entire; IR, irregular; N, no; R, regular; Rad, radical mastectomy; Reg, regional mastectomy; S, spayed; Y, yes

TABLE 3: Epidemiology, clinical and tumour characteristics of dogs included in group C (control)

Breed	Age (years)	Neutering status	Hormonal treatments	Regularity of oestrus	Number of malignant tumours	Tumour size (cm)	Fixation to muscles	Tumour ulceration	Clinical stage	Surgery	Histological diagnosis	Cox
Mixed	11	E	Y	R	2	2	N	N	IV	Reg	Solid carcinoma	3
Siberian Husky	14	S	N	R	1	1	N	N	I	Reg	Tubulopapillar carcinoma	3
Cocker Spaniel	8	S	N	R	1	1	Y	N	I	Rad	Adenosquamous carcinoma	3
Yorkshire Terrier	9	E	Y	IR	1	1.6	N	N	I	Rad	Lipid-rich carcinoma	3
Mixed	9	E	N	R	3	3	N	N	II	Rad	Solid carcinoma	3
Mixed	10	E	N	R	2	10	N	N	III	Rad	Complex-type carcinoma	1
Cocker Spaniel	9	E	N	IR	1	2	N	N	I	Rad	Solid carcinoma	3
Mixed	10	E	N	R	2	1	N	N	I	Rad	Adenosquamous carcinoma	3
Mixed	9	E	N	R	1	4	Y	N	II	Reg	Anaplastic carcinoma	3
Irish Setter	11	E	N	IR	2	5	N	N	IV	Reg	Comedocarcinoma	2
Toy Poodle	14	E	N	R	3	3	N	N	II	Rad	Solid carcinoma	3
Miniature Schnauzer	12	S	N	R	1	3	N	N	II	Rad	Comedocarcinoma	3
Mixed	10	E	N	R	1	3	N	N	II	Rad	Carcinoma and malignant myoepithelioma	3

E, entire; IR, irregular; N, no; R, regular; Rad, radical mastectomy; Reg, regional mastectomy; S, spayed; Y, yes

(Heller and others 2005). Several studies have demonstrated an association between Cox-2 expression and angiogenesis development of distant metastases, poor prognosis and shorter survival periods in CMTs (Queiroga and others 2005; Lavalle and others 2012). As it has also been reported (Clemente and others 2013), the authors found that the highest Cox-2 immunoexpression was observed in HM isolated neoplastic cells infiltrating the mammary gland stroma and in neoplastic cells in emboli in lymphatic vessels, which could be related to a still not elucidated influence of Cox-2 in cellular migration and mobility.

In the present study, 64.3 per cent of the dogs had local recurrence or metastases (21.4 per cent local recurrences and 42.9 per cent distant metastases) and 50 per cent of the animals died due to mammary cancer, which is in accordance with other studies (Peña and others 1998; Queiroga and others 2005). Additionally, most of the dogs that developed metastasis were those that did not receive mitoxantrone or firocoxib. These findings, together with the fact that treatment was selected in the univariate and multivariate analyses as a factor associated with OS and DFS, emphasise the need for adjuvant therapy in dogs with HM-CMTs.

The most important finding of this study is that firocoxib might prolong OS and increase DFS of dogs with grade III mammary tumours. Thus, although taking into consideration the low population included in this study, this supports the use of adjuvant therapy with firocoxib. Cox-2 enzymes play an important role in tumour cell biology, in tumour growth and in the processes of invasion and metastasis (Gately 2000). They also act through apoptosis inhibition and by modulating the production of variable angiogenic factors, such as vascular endothelial growth factor (VEGF) (Williams and others 1999; Gately 2000; Gately and Li 2004). Some studies have shown an association between Cox-2 and VEGF expression in malignant mammary tumours (Dias Pereira and others 2009; Queiroga and

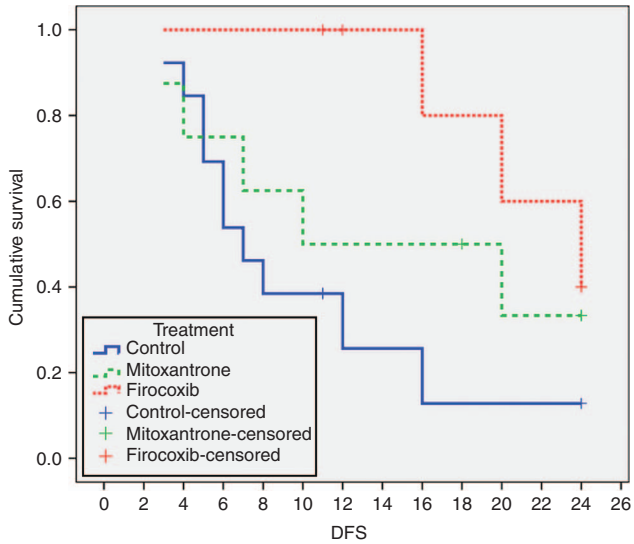

FIG 1: Kaplan–Meier disease-free survival (DFS) curve of dogs included in the study. Dogs without tumour recurrence were censored

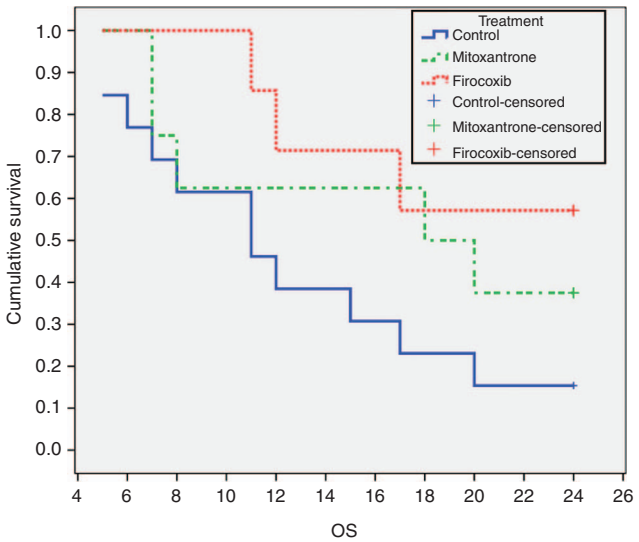

FIG 2: Kaplan–Meier overall survival (OS) curve of dogs included in the study. Dogs alive at the completion of the study were censored

others 2011; Clemente and others 2013) and more recently, it has been observed that selective Cox-2 inhibitors suppressed tumour cell growth in a CMT cell line (Saito and others 2014). In human medicine, Cox-2 inhibitors have been used successfully as part of a metronomic protocol for the treatment of advance breast cancer (Perroud and others 2013).

A previous study performed in 12 dogs with inflammatory mammary cancer showed that treatment with a non-selective Cox inhibitor (piroxicam) significantly improved the clinical condition and disease stability of dogs and also increased the OS when compared with dogs treated with doxorubicin (de Souza and others 2009). Cox inhibitors have also been used for the treatment of non-inflammatory advanced CMTs (Lavalle and others 2012), in combination (not as a single agent) with a chemotherapy (carboplatin) drug. In the later study, treatment with carboplatin alone or carboplatin with piroxicam or firocoxib after surgery led to a statistically significant longer OS when compared with surgical treatment alone. To the authors' knowledge, no studies have been performed to evaluate the efficacy of a Cox-2 inhibitor for the treatment of non-inflammatory malignant CMTs as a single agent.

The results of authors' study show that firocoxib might increase OS and DFS of dogs with HM-CMTs, when compared with control dogs and this relationship was not found when comparing dogs treated with mitoxantrone and the control dogs. This could have been due to the different clinical stage distribution between both groups, which might have been a source of bias. These results might also have been due to the different mechanisms of action of mitoxantrone and firocoxib or due to drug resistance, as this is one of the mechanisms by which chemotherapy fails to achieve cure or stable disease in some type of tumours. Because host vascular endothelial cells should be genetically stable and lack the diverse genetic defects characteristic of tumoural cells that lead to drug resistance, the anti-angiogenic effects of Cox-2 inhibitors might be more durable than those obtained with conventional chemotherapy (Kerbel and Kamen 2004).

Doxorubicin and mitoxantrone have been recommended for the treatment of dogs with advanced mammary cancer (Simon and others 2001). Mitoxantrone was chosen in authors' study as a chemotherapeutic agent as it has less toxicity than doxorubicin (Lana and Dobson 2011). It has also been shown to be of benefit in dogs with inflammatory mammary cancer (Clemente and others 2009) and in dogs with advanced disease (stage IV) after complete surgical excision of the tumours (Tran and others 2014). Both agents, mitoxantrone and doxorubicin, inhibit topoisomerase enzymes required for DNA replication; however, doxorubicin also intercalates DNA, inhibiting protein synthesis and promoting free radical formation. On the other hand, some studies have shown that the in vitro anti-tumoural activity of doxorubicin is lower than that of the platinum agents (Simon and others 2001). A different outcome and results could have been obtained in authors' study if a drug with a wider spectrum of anti-neoplastic activity, such as doxorubicin would have been used instead of mitoxantrone or a drug with a different mechanism of action like carboplatin.

Likewise, a total of five cycles of chemotherapy was administered and, perhaps, a prolonged period of administration might give different results. Further studies with a larger population of dogs are needed to establish whether a Cox-2 inhibitor is more effective than a chemotherapeutic drug, such as mitoxantrone or a platinum agent for the treatment of HM-CMTs.

Mitoxantrone in authors' study was associated with bone marrow suppression and gastrointestinal toxicity, as it has been previously described (Lana and Dobson 2011). Toxicity was severe enough to decide to discontinue chemotherapy in one case. No severe side effects associated with long-term administration of firocoxib were observed, as previously reported (Autefage and others 2011; Knapp and others 2013). The difference between mitoxantrone and firocoxib regarding side effects (75 per cent v 28 per cent respectively) is an advantage for its use as adjuvant treatment for malignant CMTs.

There are several limitations within the present study, which must be considered in the interpretation of these results. The small number of dogs included in each group is the main limitation; the treatment allocation system was performed in cases (in groups A and B) but not in controls, as this decision was made by the owners. Also, the distribution of clinical stages between groups was not equal, as a greater proportion of dogs in the mitoxantrone treatment group had stage IV disease. Although the difference regarding the distribution of clinical stage between groups was not statistically different, this could have led to the differences found between groups. A randomised study with a greater number of cases comparing the efficacy of chemotherapy versus Cox-2 inhibitors as adjuvant treatment would be more appropriate to assess these differences.

In conclusion, this study confirms that postsurgical adjuvant therapy might increase DFS and OS of dogs with HM-CMTs. Firocoxib alone reduces the proportion of tumour recurrences or metastases during follow-up. The low toxicity and the high OS and DFS achieved in dogs treated with firocoxib warrant the use of Cox-2 inhibitors for the treatment of HM-CMTs. Future studies including larger number of patients, especially with clinical stages III and IV, are needed to compare the efficacy of chemotherapy drug versus Cox-2 inhibitors as adjuvant treatment in these cases.

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Adjuvant therapy for highly malignant canine mammary tumours: Cox-2 inhibitor versus c hemotherapy: a case–control prospective study

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