Canine indolent and aggressive lymphoma: Clinical spectrum with histologic correlation

(Article begins on next page)

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14   **A retrospective analysis of cChemotherapy switch suggests improveds outcome in**

15   **surgically removed, biologically aggressive canine haemangiosarcoma**

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27   **Short title**: chemotherapy switch in aggressive haemangiosarcoma

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32   Findings of this study were presented in part at the European Society of Veterinary Oncology

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Abstract

Haemangiosarcoma (HSA) has an aggressive biological behaviour and carries a poor prognosis, with less than 10% of treated dogs surviving longer than one year.

In this retrospective study a varied metronomic chemotherapy (MC) regimen preceded by ~~standard~~ adjuvant doxorubicin-based maximum-tolerated dose chemotherapy (MTDC) was compared to MTDC, in terms of efficacy (time to metastasis, TTM, and survival time, ST) and safety in dogs with biologically aggressive HSA. Dogs were eligible if they had no metastasis after MTDC and received either no further chemotherapy or MC maintenance.

~~Twenty-two dogs were enrolled: 12~~ Twelve dogs received MTDC, and 10 received MC thereafter. Median TTM and ST were significantly longer for dogs receiving MTDC-MC (not reached versus 150 days, P=0.028; and not reached versus 168 days, P=0.030, respectively). Treatment was well tolerated.

MTDC followed by MC is safe and suggests ~~improved~~ improve TTM and ST in dogs with surgically removed, biologically aggressive HSA that are treated in the microscopic setting.

3

Introduction

Haemangiosarcoma (HSA) is a common mesenchymal tumour in dogs, arising in three different forms: dermal, subcutaneous/muscular and visceral, the latter mainly involving spleen, right atrium or auricle, and liver.[1-3] With the exception of the dermal form, which may behave in a less aggressive fashion, subcutaneous/intramuscular and visceral HSA is a highly malignant cancer, spreading rapidly to lungs, liver, peritoneum and central nervous system.[4,5] Unfortunately, visceral HSA has a silent evolution for a quite long time, and is accompanied by non specific clinical signs. As a consequence, when detected, it is usually in an advanced or metastatic stage, therefore precluding cure.[1,2]

The mainstay of treatment consists of surgery followed by adjuvant intravenous chemotherapy.[6,7] Doxorubicin-based chemotherapy protocols have been administered to dogs with HSA, including doxorubicin as single agent,[6] or combined with ifosfamide,[8] vincristine and cyclophosphamide,[7,9-11] and epirubicin as single agent.[12] Although a three weekly regimen is the commonest schedule administration of doxorubicin, one study attempting to increase dose intensity by more frequent administrations showed such strategy to be well tolerated; however, survival time was not improved.[13]

Although the combination of doxorubicin and dacarbazine has provided promising results in a recent clinical trial, it is still common knowledge that < 10% of the dogs diagnosed with HSA will survive one year after diagnosis, being attributable to the development of metastatic disease during or after completion of maximum-tolerated dose chemotherapy (MTDC).[14] Thus, it appears obvious that MDTC is unlikely to provide a durable response in such biologically aggressive solid tumours.

Metronomic chemotherapy (MC) refers to the frequent administration of cytotoxic drugs at doses significantly lower than the maximum tolerated dose, with no prolonged drug-free breaks, leading to an anti-angiogenic effect and immune-modulation.[15-16] In veterinary oncology, MC has been mainly used in a palliative setting with good

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83 response rates and safety profile.[17-18] A continuous low-dose chemotherapy strategy

84 consisting of cyclophosphamide, etoposide, and piroxicam has been proposed as an

85 alternative treatment for dogs with HSA, yielding comparable results to conventional

86 MTDC, therefore suggesting a beneficial effect of this regimen in delaying disease

87 progression in canine HSA.[19] A more recent study suggested that the combination of

88 both MTDC and MC was more efficacious in dogs with splenic HSA than either type of

89 chemotherapy alone in the early follow-up period; however, no significant prolongation

90 of survival time was observed during the late follow-up period when compared with

91 dogs undergoing splenectomy only.[20]

92 A "chemo-switch schedule" refers to the introduction of a new and potentially non–

93 cross-resistant agent after completion of first-line chemotherapy, such as the

94 administration of MC after MTDC.[21] In the current study, we retrospectively compared

95 MC preceded by doxorubicin-based MTDC to MTDC treatment only, in terms of

96 efficacy (time to metastasis, TTM, and survival time, ST) and safety in dogs with

97 biologically aggressive HSA. It was hypothesised that chemo-switch would improve

98 long-term tumour control.

99

100

101 **Material and methods**

102

103 *Inclusion criteria*

104

105 The databases of the Centro Oncologico Veterinario (Bologna, Italy), Centro

106 Veterinario Berna (Lisbon, Portugal) and University of Milan Teaching Hospital

107 (Milan, Italy) were reviewed to identify client-owned dogs with histologically

108 confirmed and biologically aggressive HSA (2011-2014).

109 Haemangiosarcoma was considered as "biologically aggressive" if arising from any

110 visceral, bone and muscular location or, in case of subcutaneous tumours, if the largest

111 diameter was > 6 cm.[1-4]

112 Eligible dogs for inclusion in the analysis set were those that had no evidence of

113 macroscopic disease after completion of MTDC based on imaging and that received

114 either no further chemotherapy or MC maintenance.

115 Pre-surgical, pre-dosing, and post-dosing investigations included physical examination,

116 haematology, serum biochemistry, abdominal ultrasound and at least two lateral views

117 thoracic radiographs or computed tomography (CT) if performed.

118 Dogs were monitored at least every three months after MTDC or during MC

119 maintenance, as listed above.

120 Dogs were staged according to the World Health Organization (WHO) staging system

121 for domestic animals.[22]

122

123 *Treatment protocol*

124 Based on owners' and clinicians' preference, dogs received MTDC followed by MC

125 (Group 1) or MTDC only (Group 2). MTDC consisted of a discontinued doxorubicin-

126 based chemotherapy protocol. MC was administered orally and consisted of low-dose

127 cyclophosphamide (Endoxan®, Baxter s.r.l., Lurago d'Erba, Como, Italy) administered

128 q24h or q48h at 7-15 mg/m^2, and the cyclooxygenase-2 (COX-2) inhibitor firocoxib

129 (Previcox®, Merial, Lyon, France), meloxicam (Metacam®, Boehringer Ingelheim,

130 Milan, Italy), or a non-selective COX inhibitor (Piroxicam®, Pfizer Italia s.r.l., Latina,

131 Italy) administered daily at the standard recommended dose. The non-steroidal anti-

132 inflammatory drug (NSAID) varied depending on clinician's preference. In case of

133 haemorrhagic cystitis, cyclophosphamide was discontinued and dogs received oral

134 chlorambucil (Leukeran®, GlaxoSmithKline S.p.A., Verona, Italy) at the dosage of 4

135 mg/m^2 q24h or q48h.[23]

136 Depending on availability, oral thalidomide at 2-3 mg/kg (Thalidomid, Bichsel AG,

137 Interlaken, Switzerland) was also administered q24h or q48h depending on clinician's

138 preference. The dose of thalidomide was arbitrarily chosen based on some of the

139 authors' experience.[24] Owners intending to have thalidomide administered were

140 informed on its known teratogenic effect.[25]

141

142 *Assessment of toxicity*

143 Toxicity resulting from MTDC was assessed in both groups based on the dog's history,

144 physical examination and complete blood counts (CBCs) 7-10 days after chemotherapy

145 and before the beginning of each next cycle, as stated by the Veterinary Co-operative

146 Oncology Group.[26] In Group 2, urinalysis was also carried out in the case of suspected

147 urothelial toxicity (i.e. haematuria, stranguria, pollachiuria).

148

149 *Statistical analysis*

150 Follow-up and survival times were calculated from the date of diagnosis to the date of

151 last visit or death. For both groups, ST and TTM (beyond regional lymph nodes) were

152 explored with the Kaplan-Meier product limit method followed by log-rank test. In

153 either group, timing was considered from surgical excision. In the survival analysis,

154 dogs were censored if they were alive at the time of data accrual closure or died of no

155 tumour-related causes, whereas for TTM dogs were censored if, by the last examination,

156 distant metastases had not developed.

157 Causes of death were established reviewing the individual dog clinical histories and

158 through telephone calls to owners and referring veterinarians. Dogs were considered to

159 have died of HSA if the clinical staging work-up was consistent with the presence of

160 metastatic disease and if symptoms could be linked to HSA progression (i.e recurrence

161 of haemoabdomen); dogs were considered not to have died because of HSA if their last

162 staging work-up (performed no longer than one month before death) revealed no

163 evidence of metastatic disease and if death was determined to occur due to an unrelated

164 cause.

165 When appropriate, data sets were tested for normality by use of the D'Agostino and

166 Pearson omnibus normality test. Values were expressed as mean ± standard deviation in

167 case of normal distribution, or as median with a range in case of non-normal

168 distribution.

169 To verify whether features of the two groups differed at admission or during MTDC, the

170 T-test (parametric variables) or Mann Whitney U test (non-parametric variables) was

171 used to compare age, body weight, and the time occurred from the diagnosis to the

172 beginning of MTDC. Fisher's exact test was used to compare breed (pure- vs cross-

173 breed), sex (male vs female), primary location of the tumour (spleen vs other sites),

174 clinical stage, number of doxorubicin cycles (<4 vs 4-6), type of chemotherapy protocol

175 (single agent doxorubicin vs poly-chemotherapy) and MTDC-related toxicity (present

176 vs absent). Data were analysed by use of commercial software programs (SPSS

177 Statistics v. 19, IBM, Somers, NY, and Prism v. 5.0, GraphPad, San Diego, CA). P

178 values ≤ 0.05 were considered significant.

179

180

181 **Results**

182 Twenty-two dogs met the inclusion criteria and were ~~enrolled~~included in the analysis; 10

183 (45.5%) of them received MTDC followed by MC (Group 1), whereas the remaining 12

184 (54.5%) were treated with MTDC (Group 2). Dogs' characteristics are listed in Table 1. Dogs

185 were not stratified based on prognostic risk, but there was good balance between arms

8

186 regarding dogs' features and possible outcome variables; however, concerning sex
187 distribution, there was a statistically significant difference between groups, as males were
188 more common in Group 1 and females were more common in Group 2 (P=0.043; Table 2).
189 For all dogs, pre-surgical, pre-dosing, and post-dosing imaging investigations were performed
190 through thoracic radiographs and abdominal ultrasound. Two dogs (case 6 and case 22; Table
191 1) had CT scans repeated throughout the follow-up period.

192

Group 1 (MTDC-MC)

194 There were 3 mixed breed dogs, 2 German shepherds, 1 Golden retriever, 1 Labrador
195 retriever, 1 Boxer, 1 Great Dane, and 1 Italian cane Corso. Mean age was 8.9 ($\pm$ 2.6)
196 years and mean weight was 36.4 ($\pm$ 12.0) kg. There were 8 males (n=4 neutered) and 2
197 spayed female dogs. HSA occurred in the spleen as primary site in 8 dogs; all dogs
198 presented with hemoperitoneum because of splenic rupture. The remaining 2 dogs had
199 subcutaneous (n=1) and osseous (n=1) HSA.

200 All dogs underwent surgery, consisting of splenectomy, removal of the subcutaneous
201 tumour, or amputation according to cancer location. Histopathological evaluation
202 revealed clean surgical margins in the subcutaneous and osseous HSA; surgical margins
203 were deemed not assessable for dogs presenting with visceral rupture.

204 According to the WHO classification, 9 dogs had stage II disease, and 1 dog with
205 osseous HSA had stage III disease.

206 The mean time from surgery to initial MTDC administration was 20.8 ($\pm$ 15.4) days.
207 Eight dogs received doxorubicin as single agent, and 2 dogs received a combination of
208 doxorubicin and dacarbazine. For all dogs, the median number of doxorubicin cycles
209 was 5 (range, 4 to 6 cycles), and the initial dose was 30 mg/m^2 for all dogs.
210 Chemotherapy dose reduction was undertaken in 3 dogs receiving single agent

211   doxorubicin; this was performed at the clinician's discretion after haematological and/or

212   gastrointestinal toxicity developed: 2 dogs had 10% and 1 had 20% dose reduction. The

213   median total dose of doxorubicin was 132 mg/m^2 (range, 120 to 180 mg/m^2).

214   The median time from completion of MTDC to start of MC was 17.5 days (range, 13 to

215   24 days). Cyclophosphamide was administered q24h in 2 dogs and q48h in the

216   remaining 8 dogs. The median single cyclophosphamide dose was 8.5 mg/m^2 (range, 7

217   to 15 mg/m^2), and the median weekly cumulative dose was 44 mg/m^2 (range, 28 to 105

218   mg/m^2). Concerning NSAIDs, 5 dogs received piroxicam, 4 had meloxicam and 2 dogs

219   received firocoxib. Thalidomide was given in combination with standard MC in 7

220   (70%) of 10 dogs: 5 dogs received 2 mg/kg q24h, whereas the remaining 2 were treated

221   at 3 mg/kg q24h.

222

223   ***Group 2 (MTDC)***

224   There were 5 mixed breed dogs, 2 Labrador retriever and 1 each of Boxer, German

225   shepherd, Pitt Bull, Rottweiler and Yorkshire terrier. Mean age was 9.8 (± 2.2) years

226   and mean weight was 27.2 (± 10.4) kg. There were 8 female (n=4 spayed) and 4 males

227   (n=1 neutered) dogs. HSA occurred in the spleen as primary site in 11 dogs; 10 of them

228   presented with hemoperitoneum because of splenic rupture. One dog had a

229   subcutaneous HSA.

230   All dogs underwent surgery, consisting of splenectomy and removal of the

231   subcutaneous tumour according to cancer location. Histopathological evaluation

232   revealed clean surgical margins in the subcutaneous HSA; surgical margins were

233   deemed not assessable for dogs presenting with visceral rupture.

234   According to WHO, 11 dogs had stage II disease, and 1 had stage I disease. The dog

235   with stage I disease had a splenic HSA.

236 The mean time from surgery to initial MTDC administration was 25.0 (± 12.1) days.

237 Nine dogs received doxorubicin as single agent and 3 dogs received a combination of

238 doxorubicin and dacarbazine. The median number of doxorubicin cycles was 4 (range, 2

239 to 5 cycles) and all dogs received a starting dose of doxorubicin of 30 mg/m^2.

240 Chemotherapy dose reduction was performed in 2 dogs receiving single agent

241 doxorubicin; this was performed at the clinician's discretion due to haematological

242 and/or gastrointestinal toxicity: one dog had 10% and one had 20% dose reduction. The

243 median total dose of doxorubicin was 120 mg/m^2 (range, 60 to 180). In the three dogs

244 receiving doxorubicin and dacarbazine, the protocol was designed as previously

245 reported.[14] Cases' data are summarized in Table 1.

246

247 ***Clinical outcome***

248 Three (30%) out of the 10 dogs included in Group 1 (MTDC-MC) developed metastatic

249 disease after 119, 151 and 460 days, respectively. Metastases were found in the

250 peritoneum (n=2) and liver and lung (n=1). The two dogs with metastases to the

251 peritoneum developed haemoabdomen.

252

253 Nine (75%) of the 12 dogs included in Group 2 (MTDC) developed metastatic disease

254 after a median of 134 days (range, 89 to 174 days). Metastases were found in lung

255 (n=3), peritoneum (n=2), liver (n=2), lung and brain (n=1) and lung, stomach and liver

256 (n=1). The two dogs with metastases to the peritoneum developed haemoabdomen.

257

258 Overall, median TTM was significantly longer for dogs receiving MTDC-MC compared

259 to those receiving MTDC only (not reached versus 150 days, respectively; P=0.028;

260 Figure 1).

261

11

262   Six (60%) out of the 10 dogs included in Group 1 (MTDC-MC) were dead at the end of

263   the study. Three (27.2%) dogs with splenic HSA died as a result of disease progression

264   after 152, 191 and 487 days. Three dogs with splenic HSA died of tumour-unrelated

265   causes after 165, 292 and 730 days, respectively, with no evidence of tumour recurrence

266   or metastasis. One dog (splenic HSA) was lost to follow-up after 680 days from the

267   diagnosis; at the last visit this dog had no evidence of macroscopic disease.

268   Three dogs (osseous, n=1, and splenic, n=2) were still alive with no evidence of disease

269   after 311, 640 and 1280 days, respectively.

270

271   Ten (83.3%) out of the 12 dogs in Group 2 (MTCD) were dead at data analysis closure:

272   9 (75%) died as a result of HSA progression with a median survival time of 156 days

273   (range, 97 to 341 days). Of these 9 dogs, 7 had splenic stage II HSA, 1 had splenic stage

274   I HSA, and one had subcutaneous stage II HSA. The remaining dog (splenic stage II

275   HSA) died 803 days after the diagnosis because of tumour-unrelated causes.

276   Two dogs with splenic HSA were still alive with no evidence of disease at 437 and 608

277   days, respectively.

278

279   Overall, dogs receiving MTDC followed by MC had a significantly longer median ST

280   than those receiving MTDC only (not reached versus 168 days, respectively; P=0.030;

281   Figure 2).

282

283   *Toxicity*

284   During MTDC, neutropenia occurred in 4 (40%) dogs in Group 1. One dog had one

285   episode of grade 1 neutropenia, 2 dogs had one episode of grade 2 neutropenia, whereas

286   1 dog had 2 episodes of grade 2 neutropenia. In all dogs haematological toxicities

287   resolved without sequel.

288 In Group 2, 1 (8.3%) dog developed 2 episodes of grade 4 non-febrile neutropenia, and

289 1 (8.3%) dog developed one episode of grade 2 anaemia.

290 Gastrointestinal toxicity was the second most common adverse event in both groups,

291 and consisted of vomiting, diarrhoea and decreased appetite of mild to moderate

292 severity. Gastrointestinal toxicity of grade 2 occurred in 2 (20%) dogs in Group 1.

293 These dogs had no concurrent episodes of haematological toxicity. In Group 2, 3 (25%)

294 dogs developed gastrointestinal toxicity: 1 dog had one episode of grade 3 anorexia and

295 2 dogs had 1 episode of grade 2 vomiting (1 concurrently had grade 2 anaemia).

296 The overall frequency of MTDC related side effects did not differ between groups

297 (Table 2).

298

299 During MC, 4 (40%) dogs developed gastrointestinal, haematological and/or urothelial

300 adverse events. Two dogs developed grade 2 sterile haemorrhagic cystitis after 180 and

301 470 days, respectively; in both cases cyclophosphamide was discontinued and

302 chlorambucil was started; cystitis resolved within 4 weeks in both cases. One dog

303 developed grade 1 diarrhoea and in one case grade 1 vomiting and diarrhoea occurred

304 simultaneously. Gastrointestinal signs resolved with symptomatic treatment and did not

305 recur.

306

307 **Discussion**

308 The treatment of HSA continues to be extremely challenging in veterinary oncology.

309 Unfortunately, little progress has been made over the years, and prognosis for dogs with

310 HSA is poor as a result of the aggressive nature of the disease, leading to invasion of

311 nearby organs and vessels, early metastasis and limited treatment options providing

312 durable disease control. Surgery is designed to remove all macroscopic tumours and

313 prevent further risk of acute haemorrhage, but is considered purely palliative. The

13

314 addition of chemotherapy in an effort to treat microscopic disease has been documented

315 to provide a modest improvement in outcome, with reported median survival times in

316 the range of 6-8 months and less than 10% of dogs being alive at 12 months.[1,2]

317 The "cell kill" paradigm associated with MTDC has been successful in the treatment of

318 human and canine haematological neoplasia, but unfortunately this has not provided

319 long-lasting responses in the majority of advanced solid tumours.[21] Failure of MTDC

320 may be multifactorial, being attributable to the heterogeneity of cancer cells, genetic

321 make-up, and the influence of tumour microenvironment, thereby giving rise to

322 treatment resistance.[21] Based on the above, the Gatenby's hypothesis of controlling

323 tumour growth instead of trying to eradicate it may become a more rational strategy.[27]

324 Maintenance therapy refers to a treatment that is given to avoid disease progression

325 after the cancer has been successfully controlled with the initial therapy.[21]

326 An effective maintenance therapy should accomplish good patient tolerability, lack of

327 cumulative toxicities, and cost–effectiveness. Maintenance therapy may consist of

328 "continuation" therapy where one drug of the initial therapy is continued after the

329 induction phase of the protocol, or of "switch" maintenance in which a new agent is

330 introduced.[21,28]

331 Switch maintenance has been recently investigated in canine stage I-II splenic HSA by

332 administering the tyrosine kinase inhibitor toceranib phosphate. Toceranib mainly

333 targets the stem cell factor receptor KIT, platelet derived growth factor receptor and

334 vascular endothelial growth factor receptor (VEGFR), which are typically expressed by

335 canine HSA.[29]

336 As in our study, the switch maintenance was administered in the microscopic disease

337 setting after completion of doxorubicin MTDC. Unfortunately, disease-free interval nor

338 ST were improved when comparing dogs receiving or not receiving maintenance

339 toceranib.[29]

It has become progressively clear that the endothelial cell compartment is an attractive target for anticancer therapy as a result of the evident importance of the tumour vasculature for sustaining tumour growth and metastasis. Also, the endothelial cells are sensitive to the action of conventional cytotoxic drugs, including cyclophosphamide, if the dosing regimen is altered to the so-called anti-angiogenic scheduling.[15]

In a previous study, dogs with stage II HSAAS receiving an oral adjuvant therapy consisting of alternating low-dose daily cyclophosphamide and etoposide in combination with piroxicam had comparable survival times to historical controls treated with conventional doxorubicin chemotherapy.[19] Starting from the promising results of the mentioned study, Based on the promising results obtained by MC in the treatment of surgically-removed canine HSA,[19] we hypothesised that outcome might be improved, if a MC schedule is to be administered after MTDC as a consolidation strategy. To this end, we retrospectively compared HSA dogs receiving MTDC versus MTDC followed by MC in the microscopic setting. Beside cyclophosphamide and NSAID, thalidomide was added to this combination in the majority of dogs.

The results obtained in the current study document suggest an advantage of the addition of maintenance MC over MTDC alone in terms of metastatic control and survival. Indeed, dogs undergoing chemo-switch after dose-intense chemotherapy had a significantly longer TTM and ST compared to dogs receiving MTDC, suggesting that chemo-switch improves long-term tumour control in biologically aggressive canine HSA. These results can may be explained by the following considerations.

The use of continuous, low-dose cyclophosphamide exerts potent anti-angiogenic properties through the inhibition of proliferation and/or induction of apoptosis of activated endothelial cells, selective inhibition of migration of endothelial cell, increase in the expression of thrombospondin-1, and sustained decrease in levels and viability of bone marrow-derived endothelial progenitor cells.[15] Moreover, it has been shown that

15

366   metronomic cyclophosphamide can also target the immune system by activating or

367   restoring its antitumor properties, particularly through the inhibition of T regulatory

368   lymphocytes and enhance the cytotoxic T lymphocytes response.[30,31]

369   Non-selective NSAIDs and COX-2 selective inhibitors such as piroxicam and

370   meloxicam are effective in counteracting tumour angiogenesis, by boostering the effect

371   of cyclophosphamide.[32-34]

372   Alongside its teratogenic effect, thalidomide is a potent inhibitor of angiogenesis

373   through inhibition of VEGF, basic fibroblastic growth factor, and tumour necrosis factor

374   alpha, and may play a role in anti-angiogenic strategies.[35]

375   A recent study has suggested that the combination of MTDC and MC may be superior

376   to MTDC alone in the treatment of canine splenic HSA in the early follow-up period,[20]

377   however survival times were modest compared to the group receiving MTDC alone and,

378   importantly, these did not differ substantially from the published literature.[1,2] In the

379   aforementioned study, 13 dogs with splenic HSA received doxorubicin and MC either

380   sequentially (chemo-switch; n=6) or concurrently (n=7). Median survival time for these

381   dogs was 4.3 months, and median duration of treatment was 56 days; it was

382   hypothesised that metastatic disease rapidly progressed after chemotherapy was

383   interrupted for whichever reason.[20] In the current study group, the use of MC

384   significantly improved outcome, and it may be hypothesised that the difference between

385   our study and Wendelburg's study may be due to the use of the potent antiangiogenic

386   drug thalidomide or to the continuous use of MC.

387   While MTDC can serve to de-bulk HSA by directly targeting the cancer cells,

388   maintenance MC may disrupt crucial angiogenic pathways, impeding the inevitable

389   rebound and regrowth, ultimately translating into significant therapeutic benefits.

390   In agreement with previous studies, MC was well tolerated, and side effects were

391   mainly gastro-intestinal and of mild severity.[17,18] Haemorrhagic cystitis occurred in 2

dogs, most likely as a consequence of prolonged treatment with cyclophosphamide; however gastrointestinal and haematological adverse event could have also been due to transient and undiagnosed comorbidities and not related to MC.

Limitations of this study include its retrospective nature, the low number of cases, the different tumour site origin, the variability of chemotherapy protocols used in the MTDC phase and the lack of necropsies. Five dogs received a combination of doxorubicin and dacarbazine, which has recently demonstrated encouraging results providing an increase in the chances of survival for biologically aggressive canine HSA.[14,36] Nevertheless, in the present series dogs receiving doxorubicin and dacarbazine were equally distributed among groups, thereby rendering unlikely the chance of having improved outcome in one group only. Dogs' features and possible outcome variables were homogeneously distributed between groups with the exception of sex: male dogs were more common in Group 1 whereas females were more common in Group 2. Although this finding is likely to be a bias due to the small sample size, we cannot exclude that the small number of dogs included in this study may have contributed to reach significance for the other variables analysed.

This finding, although statistically significant, is likely to be a bias due to the small sample size, and any other explanation might only be speculative.

Finally, it must be acknowledged that 3 dogs treated with MTDC were censored belatedly (after 437, 608 and 803 days), compared to 6 dogs treated with MTDC and MC, and among them 3 were censored early (after 165, 292 and 311 days). While this may reflect a better outcome, as fewer dogs died due to HSA in Group 1 compared to Group 2, it also could have biased the results, as early deaths due to tumour-unrelated causes may strongly influence statistics.

To conclude, maintenance MC is well tolerated and may prolong TTM and survival time in dogs with biologically aggressive HSA with negative staging after completion of MTDC. Although the role of thalidomide in the treatment of HSA needs further studies, it is possible that this drug used in combination with standard MC plays an important role in controlling the metastatic process of biologically aggressive canine HSA. Prospective studies with larger number of patients are required to confirm these findings.

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550 **Captions to figures:**

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552 **Figure 1**: Time to metastases for dogs treated with MTDC-MC (dots) and MTDC (line).

553 In the MTDC-MC group, dogs had a longer time to metastases (not reached versus 150

554 days, respectively; P=0.028).

555

556 **Figure 2**: Survival time for dogs treated with MTDC-MC (dots) and MTDC (line). In

557 the MTDC-MC group, dogs had a longer survival time (not reached versus 168 days,

558 respectively; P=0.030).

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