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14 **A retrospective analysis of chemotherapy switch suggests improved outcome in**
15 **surgically removed, biologically aggressive canine haemangiosarcoma**

16
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26
27 **Short title:** chemotherapy switch in aggressive haemangiosarcoma

28
29 **Keywords:** haemangiosarcoma, metronomic, thalidomide, chemotherapy switch, dog

30
31
32 Findings of this study were presented in part at the European Society of Veterinary Oncology
33 Annual Meeting, Krakow, Poland, 2015.

34
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39 **Abstract**

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

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

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

52 MTDC followed by MC is safe and suggests improveds TTM and ST in dogs with
53 surgically removed, biologically aggressive HSA that are treated in the microscopic
54 setting.

55

56

57 **Introduction**

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

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

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

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

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/m², 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/m² 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 \pm 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

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

193 ***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/m² 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/m² (range, 120 to 180 mg/m²).

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/m² (range, 7
217 to 15 mg/m²), and the median weekly cumulative dose was 44 mg/m² (range, 28 to 105
218 mg/m²). 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 (\pm 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/m².
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/m² (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

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

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

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

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

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

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

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 boosting 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

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

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

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

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

416

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

424

425

426 **References**

427

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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; $P=0.030$).

559