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Outcome of adjuvant chemotherapy with lomustine, vinblastine and chlorambucil on management of canine mast cell tumour of high to intermediate risk

[*Resultado da quimioterapia adjuvante com lomustina, vimblastina e clorambucil no manejo do mastocitoma canino de risco alto a intermediário*]

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ABSTRACT

In spite of the many available protocols, the use of chemotherapy for the management of canine mast cell tumours (MCT) remains empirical, and there is lack of criteria for the choice of protocol and definition of patients who may benefit from treatment. The objective of this study was to evaluate the outcome of dogs with MCT after adjuvant chemotherapy according to the risk of recurrence or metastasis proposed on the literature. This prospective study included 89 followed up dogs with prognosis assesment including clinical, histological, immunohistochemical and genetic features of canine MCT. Patients were grouped according to risk of recurrence and metastasis and recommended treatment with lomustine followed by chlorambucil if considered at high-risk, or vinblastine followed by chlorambucil if a patient was at intermediate risk. Outcome was defined by disease-free interval (DFI) and overall survival (OS) estimated by Kaplan-Meier curve. Adjuvant lomustine was useful for control of canine MCT of high-risk of recurrence or metastasis, but only when sequentially associated to chlorambucil with a DFI of 686 days and not reached OS. There was no difference in outcome in the intermediate-risk group despite choosen treatment. Patients at intermediate-to-low risk may not require adjuvant treatments, even in the absence of free surgical margins.

Keywords: dog, neoplasm, mast cells, Ki-67, c-kit

RESUMO

Apesar dos inúmeros protocolos disponíveis, o uso da quimioterapia permanece empírico para o mastocitoma canino e faltam critérios para escolha do protocolo e da definição dos pacientes que poderiam se beneficiar do tratamento. O objetivo deste estudo foi avaliar o resultado de cães com mastocitoma após a quimioterapia adjuvante, de acordo com o risco de recorrência ou metástase proposto na literatura. Este estudo prospectivo incluiu 89 cães com acompanhamento clínico e avaliação prognóstica, incluindo características clínicas, histológicas, imuno-histoquímicas e genéticas dos mastocitomas. Os pacientes foram agrupados segundo o risco de recorrência ou metástase, sendo recomendado tratamento com lomustina seguida de clorambucila, se considerados sob alto risco, ou vimblastina seguida de clorambucila, se estivessem sob risco intermediário. O resultado final foi definido pelo intervalo livre de doença (ILD) e pela sobrevida global (SG), estimados pela curva de Kaplan-Meier. Na adjuvância, a lomustina foi útil no controle do mastocitoma canino de alto risco, mas apenas quando associada ao clorambucila, com um ILD de 686 dias, sem atingir a mediana para SG. Não houve diferença no grupo de risco intermediário, independentemente do tratamento escolhido. Pacientes de risco intermediário podem não necessitar de tratamentos adjuvantes, mesmo na ausência de margens cirúrgicas livres.

Palavras-chave: cão, neoplasia, mastócitos, Ki-67, c-kit

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INTRODUCTION

An increasing focus is given to canine mast cell tumour (MCT), due to its high prevalence in veterinary oncology. Although most low-to-intermediate grade MCT may be treated with surgery alone resulting in prolonged survival times, it is not uncommon to have patients who succumb to this neoplasm, which can manifest itself in an extremely aggressive and fatal form (Dobson and Scase 2007).

A high risk of recurrence or metastasis is associated with tumours classified as a grade 3 MCT according to the Patnaik grading scheme, but also for the Patnaik intermediate grade, whenever there is a lymph node or distant metastasis and / or high growth fraction estimated by the mitotic count or Ki-67 immunoexpression (Romansik *et al*., 2007; Blackwood *et al*., 2012; London and Thamm, 2013; Miller *et al*., 2014), and for the MCT located in the mucousa or mucocutaneous junctions (London and Thamm, 2013). Other prognostic factors, may also be important for disease progression, such as tumour location (scrotum, prepuce, perineum or vulva), breed (London and Thamm, 2013) and KITr pattern expression (Kiupel *et al*., 2004). Specific gain-of-function genetic mutations are also largely associated with worse prognosis (Zemke *et al*., 2002; Webster *et al*., 2006; Webster *et al*., 2008; Avery, 2012).

Radiotherapy is a highly accepted adjuvant treatment for canine MCT, with an overall cure rate of 90%, although such outcome may be related to the studied population (Frimberger *et al*., 1977, Al-Sarraf *et al*., 1996). Adjuvant chemotherapy for canine MCT is historically of limited use, however, considering the copious variation of the biological behaviour of this neoplasm, medical therapy has been indicated as an adjuvant treatment for metastatic or high-risk MCT (Thamm *et al*., 2006, Cooper *et al*., 2009). Chemotherapy is also used in the neoadjuvant setting, in an attempt to promote cytoreduction to facilitate the surgical excision (Rassnick *et al*., 1999; Thamm *et al*., 1999; Cooper *et al*., 2009; Warland *et al*., 2015), or as a sole therapy, for unresectable tumours (Taylor *et al*., 2009), although prognosis for these patients remain poor (London and Thamm, 2013). Recent studies have evaluated numerous chemotherapy protocols, such as: glucocorticoids, vinblastine, lomustine, cyclophosphamide and chlorambucil, in an adjuvant setting, for patients with advanced-stage MCT or with a high-risk of metastatic disease, however, since most studies suffered from the lack of control groups, the true benefit can not be assessed (Thamm *et al*., 2006; Cooper *et al*., 2009). Although mostly employed to patients with systemic or high risk disease, the use of adjuvant chemotherapy as part of the local control of canine MCT is not unusal, given the difficulty of obtaining free surgical margins in certain locations, limited access to radiotherapy and the absence of well established veterinary radiotherapy services in developing or undeveloped countries (Thamm *et al*., 1999; Davies *et al*., 2004).

However, despite many available protocols, the use of chemotherapy remains empirical, due to lack of controlled studies (Dobson and Scase, 2007; London and Thamm, 2013), and lack of appropriate criteria for the choice of protocol and definition of patients who may benefit from treatment. A favourable outcome is reported for dogs treated with vinblastine and prednisone, but low survival rates are reported in patients with recurrent or metastatic disease (Thamm *et al*., 1999) and novel protocols are currently under investigation, representing a subject of major interest in veterinary oncology.

The objective of this study was to evaluate the clinical outcome in dogs with MCT after adjuvant chemotherapy according to the risk of recurrence or metastasis proposed on the literature.

MATERIAL AND METHODS

This prospective study was approved by the Ethics Committee on Animal Use of Universidade Federal de Minas Gerais (protocol n° 384/2013). It included 89 dogs with histological diagnosis of cutaneous or subcutaneous MCT, without any signs of gross disease after surgical excision. All patients were staged by means of physical exam and scar revision, abdominal ultrasound, fine needle aspiration and cytology of regional lymph nodes. Lymph node removal was recommended for further staging in patients presenting suspected metastasis on cytology. Failure in compliance to

the proposed chemotherapy regime resulted in patient's exclusion from this study.

The surgical specimens of the primary tumours were cut in longitudinal sections for paraffin embedding preparation, and slides were stained with hematoxylin-eosin and toluidine blue. Histopahological examination included grading through the systems proposed by Patnaik *et al*. (1984) and Kiupel *et al*. (2011). For subcutaneous MCT, tumour extension was not considered for grading. Lymph nodes were evaluated as proposed by Weishaar *et al*. (2014), but only overt metastases were assigned as a stage II-disease.

Sections of 4 μm were cut from a representative block for each case and collected on gelatin-coated slides. The slides were deparaffinized and rehydrated in an alcohol series. Antigenal retrieval was performed with an antigen retrieval solution (Target Retrieval Solution Citrate pH 6, Dako Cytomation) under pressurized heat (20-25 mmHg, 125 °C/2 minutos). Endogenous peroxidase was blocked by immersion in 3% hydrogen peroxide and protein blockage (Protein Block, Thermo Scientific Ultravision). Primary antibodies CD117 (policlonal, 1:800, Dako Cytomation) and MIB-1 (monoclonal, 1:25, Dako Cytomation) were incubated at 4°C, for 16 hours (overnight) for KITr and Ki-67 reactions, respectively. Secondary antibody (Advance HRP Link, Dako Cytomation) was incubated in the humidity chamber for 30 minutes and the reaction was amplified by the polymer (Advance HRP Enzyme, Dako Cytomation). The reaction was revealed with the chromogen 3,3–diaminobenzidine tetrahydrochloride (Liquid DAB + Substrat Chromogen System, Dako Cytomation) and stained with Harris hematoxylin.

The immunostaining pattern for KITr was evaluated, by counting membranar, focal or difuse cytoplasmic immunoexpression (KIT patterns I, II or III, respectively) in 100 mast cells in a 40x magnification. Each MCT was assigned with the highest staining pattern present in at least 10% of the neoplastic cell population or present in large clusters of neoplastic cells within the tumour, as described by Kiupel *et al*. (2004). Ki-67 index was determined through the percentage of positive nuclei in at least 500 neoplastic cells in 3-5 fields of high immunoreactivity (hot spots) in 40x magnification. Every nuclei with immunoreactivity evidence was considered positive for Ki-67. This approach was described by Abadie *et al*. (1999), Scase *et al*. (2006) and Strefezzi *et al*. (2009). This methodology is thought to reduce subjectivity of the evaluation. Previously tested canine MCT samples were used as positive control for KITr and Ki-67, and negative controls were obtained by replacing the primary antibody by normal serum. DNA was extracted from the paraffin embedded tumours, by the proteinase K method. Primers used for amplification of the fragment of interest through PCR were designed using the BLAST software (Basic Local Alignment Search Tool®, NCBI) and manufactured by Invitrogen, as *c-kit* F: 5'-ATCTGTCTCTCTTTTCTCCCCC-3' (sense) e *c-kit* R: 5'-TGGGGTTCCCTAAAGTCATTGT-3' (antisense). The product generated by these pair of primers had 225 base pairs (bp) in the absence of mutations (native *c-kit*). Reactions were prepared and planned in a GenPro thermocycler (BIOER Technology), with a maintenance at 95 ° C for five minutes, then 30 cycles of 94°C for 45 seconds for denaturation of DNA strands, 63°C for 45 seconds to pairing and annealing of primers and 72°C for one minute to extension, to be finally maintained at 72°C for ten minutes for molecular stabilization. The amplified material was separated by electrophoresis at 100V, with free amperage. As positive and negative controls, healthy canine skin samples and milique water were used, respectively. For this study, patients with diagnosed MCT were divided into three major groups, according to the risk of recurrence or metastasis, based on evaluated pronostic factors, relevant in the literature (Blackwood *et al*., 2012; London and Thamm, 2013; Miller *et al*., 2014), as shown in table 1.

Systemic chemotherapy with lomustine (Citostal, Bristol-Meyer Squibb), at a dosage of 60-90 mg / m² every 21 days, for 3-4 sessions, followed by an eight-week protocol with chlorambucil (Leukeran, GSK) at 4-6mg/m², every 48 hours was strongly recommended for patients at higher-risk (group A), but while some owners agreed with the whole treatment (group A1), some agreed only with the maximum tolerated dose of lomustine (group A2), and some choose no further treatment (group A3 or high-risk control).

Table 1. Risk of recurrence or metastasis for canine mast cell tumours according to relevant prognostic factors in the literature (Blackwood *et al*., 2012; London and Thamm, 2013; Miller *et al*., 2014)

Risk	Profile
Low-risk	Absence of negative prognostic factors
Intermediate-risk (prognostic factors not well established)	Mast cell tumours primarily located in the scrotum, prepuce, perineum and vulva. Compromised or exiguous histological margins
High-risk	Advanced stage (II, III or IV). Patnaik grade 3. Kiupel high grade. High mitotic count (in 10 high power fields). Internal tandem duplication in exon 11 of *c-kit* oncogene. Mast cell tumours primarily located in mucousa or mucocutaneous junctions.
One single feature was sufficient to include patient in the high or intermediate risk group.	

Patients at intermediate-risk (group B) were subdivided in three groups: B1, formed by patients treated with adjuvant vinblastine (Velban, ABL) at a dosage of 2 mg / m² every seven days, for four sessions, and then every 14 days for four more sessions followed by the eight-week chlorambucil protocol; B2, formed by patients treated with the eight-week protocol using chlorambucil; and B3 formed by patients whose owners declined any further treatment beyond surgery (intermediate-risk control).

Low-risk patients were only followed up and formed a separate group (group C). All protocols of chemotherapy included concomitant administration of prednisone (Meticorten, Schering-Plough) at a dosage of 40 mg / m^2 daily during the first seven days, followed by 25 mg / m² daily for 30 days and 25 mg / m^2 every 48 hours for 60 days.

All patients undergoing chemotherapy had complete blood counts performed after 10 days of lomustine administration, after seven days of vinblastine administration or every 30 days, for chlorambucil treatment. Serum biochemistry, performed every 30 days, for all patients receiving chemotherapy, included urea, creatinine, alkaline phosphatase (ALP), alanine transaminase (ALT), aspartate transaminase (AST), gamma-glutamyl transferase (GGT) and albumin.

The disease-free interval (DFI) and overall survival (OS) were calculated from the day of surgery. For DFI, an event was considered with local recurrence, satellite lesions, lymph node or visceral metastasis. Cytology was used to confirm diagnosis in case of progressive disease and appearance of new lesions. Distant isolated cutaneous or subcutaneous MCT, were considered "de novo" primary lesions and therefore, they did not contstitute an event. OS events were constituted only for MCT related deaths. Patients lost-to-follow-up or that died due to unrelated causes were censored from the analysis. Side effects related to the use of chemotherapy were recorded according to Veterinary Cooperative Oncology Group – Common Terminology Criteria for Adverse Events (Common..., 2011).

Statistical analysis was performed using GraphPad Prism (v.6.01). Disease-free interval and overall survival were estimated through Kaplan-Meier curve. Comparisons according to risk and treatment were performed using the longrank test of Cox-Mantel. Correlation between prognostic factors and outcome for high-risk patients was performed using Spearmann test. Significance was taken as $p < 0.05$.

RESULTS

There were 89 dogs included in this study. There were 55 females and 34 males aged from three to 16,6 years of age (mean 10,3 ± 2,8) with a mean follow-up time of 338 days. A higher prevalence of Labrador (n = 18), Poodle (n = 6) and Pit-bull Terriers (n = 5) was noted. However, cross-breeds were overrepresented with 22 dogs. Other breeds presented in the study were Cocker Spaniel (n = 4), American Cocker (n = 1), Golden Retriever (n = 3), Weimaraner (n = 1), Boxer (n = 2), Pug (n = 2), French Bulldog (n = 1), Dachshund (n = 2), Pinscher (n = 3), Schnauzer (n = 3), Brazilian Fila (n = 2),

Brazilian Terrier (n = 2), Shih-tzu (n = 1), Lhasa-apso (n = 2), Yorkshire (n = 2), Staffordshire Bull Terrier (n = 1), Shiba Inu (n= 1), Maltese (n = 1), German Shepherd (n = 1), Bernese (n = 1), Argentine Mastiff (n = 1) and Deutsche Dogge (n = 1). There was no statistical difference regarding the age, sex and breed of the studied groups. All dogs were treated and / or followed up after surgical excision of cutaneous or subcutaneous MCT with extensive prognostic factors evaluation, includindg clinical staging, surgical margins, Patnaik and Kiupel grading systems, mitotic count, Ki-67 index, KITr pattern and the mutational status of the exon 11 of the *c-kit* oncogene (Table 2). In the presence of multiple lesions, the ones with worse prognosis were considered for analysis. The overall prevalence of internal tandem duplications (ITD) in the exon 11 of the *c-kit* oncogene was low (6,7%) but they were mainly present in the high-risk group, with a relative prevalence of 17,6% and no mutations detected on patients with stage I disease.

Table 2. Prognostic factors for canine mast cell tumours according to groups divided through risk of recurrence or metastasis and treatment

Prognostic factor		High-risk				Intermediate-risk		Low-risk
		Group A1 (lomustine + eight-week chlorambucil)	Group A2 (lomustine)	Group A3 (control)	Group B1 (vinblastine + eight-week chlorambucil)	Group B2 (eight-week chlorambucil)	Group B3 (control)	Group C
		n = 11	n = 10	n = 13	n = 8	n = 22	n = 10	n = 16
Clinical staging (I-IV)	I	4(36,4%)	2(20,0%)	6(46,1%)	8(100,0%)	22(100,0%)	10(100,0%)	16(100,0%)
	II	3(27,2%)	5(50,0%)	2(15,4%)	0(0,0%)	0(0,0%)	0(0,0%)	0(0,0%)
	III	4(36,4%)	2(20,0%)	5(38,5%)	0(0,0%)	0(0,0%)	0(0,0%)	0(0,0%)
	IV	0(0,0%)	1(10,0%)	0(0,0%)	0(0,0%)	0(0,0%)	0(0,0%)	0(0,0%)
Patnaik grading system	1	0(0,0%)	0(0,0%)	0(0,0%)	0(0,0%)	3(13,6%)	0(0,0%)	3(18,8%)
	2	10(90,9%)	7(70,0%)	9(69,2%)	8(100,0%)	19(86,4%)	10(100,0%)	13(81,2%)
	3	1(9,1%)	3(30,0%)	4(30,8%)	0(0,0%)	0(0,0%)	0(0,0%)	0(0,0%)
Kiupel grading system	Low-grade	3(27,3%)	1(10,0%)	4(30,8%)	8(100,0%)	22(100,0%)	10(100,0%)	16(100,0%)
	High-grade	8(72,7%)	9(90,0%)	9(69,2%)	0(0,0%)	0(0,0%)	0(0,0%)	0(0,0%)
Mitotic Index	<5 (in 10 HPF)	7(63,6%)	4(40,0%)	5(38,5%)	8(100,0%)	22(100,0%)	10(100,0%)	16(1000,0%)
	≥ 5 (in 10 HPF)	4(36,4%)	6(60,0%)	8(61,5%)	0(0,0%)	0(0,0%)	0(0,0%)	0(0%)
Ki-67	< 1.8%	1/10(10,0%)	0(0,0%)	0/12(0,0%)	0(0,0%)	3(13,6%)	1(10,0%)	1(6,2%)
	≥ 1.8%	9/10(90,0%)	10(100,0%)	12/12(100,0%)	8(100,0%)	19(86,4%)	9(90,0%)	15(93,8%%)
KITr	I	1(9,1%)	2(20,0%)	3(23,1%)	2(25,0%)	10(45,5%)	6(60,0%)	6(%)
	II	10(90,9%)	4(40,0%)	9(69,2%)	4(50,0%)	10(45,5%)	4(40,0%)	9(%)
	III	0(0,0%)	4(40,0%)	1(7,7%)	2(25,0%)	2(9,0%)	0(0,0%)	1(%)
Exon 11 do gene c-kit	Nativo	8(72,8%)	7(70,0%)	11(84,6%)	8(100,0%)	22(100,0%)	10(100,0%)	16(100,0%)
	Deleção	0(0,0%)	0(0,0%)	2(15,4%)	0(0,0%)	0(0,0%)	0(0,0%)	0(0,0%)
	Internal tandem duplication	3(27,2%)	3(30,0%)	0(0,0%)	0(0,0%)	0(0,0%)	0(0,0%)	0(0,0%)
Surgical margins	Complete	0(0,0%)	3(30,0%)	7(53,8%)	2(25,0%)	0(0,0%)	0(0,0%)	16(100,0%)
	Exiguous	1(9,1%)	1(10,0%)	0(0,0%)	1(12,5%)	2(9,1%)	4(40,0%)	0(0,0%)
	Incomplete	10(90,9%)	6(60,0%)	6(46,2%)	5(62,5%)	20(90,9%)	6(60,0%)	0(0,0%)

HPF – high power fields

Adjuvant chemotherapy with lomustine followed by an eight-week protocol with chlorambucil was recommended in all high-risk patients (n=34), however, only 11 dogs were treated with this regime (group A1). Ten patients were only treated with lomustine as the owner declined further treatment (group A2), and 13 dogs received no adjuvant treatment despite the high-risk of recurrence and metastasis (group A3).

Vinblastine followed by an eight-week treatment with chlorambucil was performed in eight patients of the intermediate-risk group (group B1), while 22 dogs received treatment only with chlorambucil for eight weeks (group B2) and 10 did not receive any further treatment (group B3). Adjuvant treatments were not offered for low-risk patients (group C, n=16) and they were followed up every 6-8 weeks.

Local tumour recurrence was not observed in this study, even in patients with narrow (n = 9) or incomplete surgical margins (n = 53), and even in the absence of surgical margins and adjuvant therapies (n = 16/62). Disease progression was noted in 23,6% of patients (21/89), ocurring in 58,9% (20/34) of the high-risk group (nine patients without any adjuvant treatment–A3) and

2,5% (1/40) in the intermediate-risk group. MCT related-deaths ocurred in 19,1% (17/89) of patients during this study, all in the high-risk group, with two, six and nine patients in groups A1, A2 and A3, respectively. Rescue therapies were attempted in 10 of 21 patients with progressive disease with masitinib (n = 5; two in group A1, two in A2 and one in A3), toceranib (n = 1; group A3), lomustine (n = 1; group A3) or surgical excision followed by masitinibe (n = 1; group A3) or lomustine followed by chlorambucil (n = 3; equally divided between A1, A3 and B2), but only three patients (equally divided between groups A1, A3 and B2) achieved survival times longer than 180 days after disease progression.

Every progression in this study resulted in metastasis to regional lymph nodes and/or formation of multiple lesions in the skin, with 71.4% of those (15/21) resulting in ulceration. Three patients at intermediate risk developed "de novo" MCT, characterized as new independent, but solitary lesions, also associated with low to intermediate risk, after surgical excision and histopathology analysis, and those were, therefore treated as primary disease, and not as progression from the earlier diagnosed MCT.

According to the risk of recurrence and metastasis, the median was reached only in the high-risk group, with DFI and OS of 134 and 258 days, respectively, as shown in figure 1 (P <0.0001).

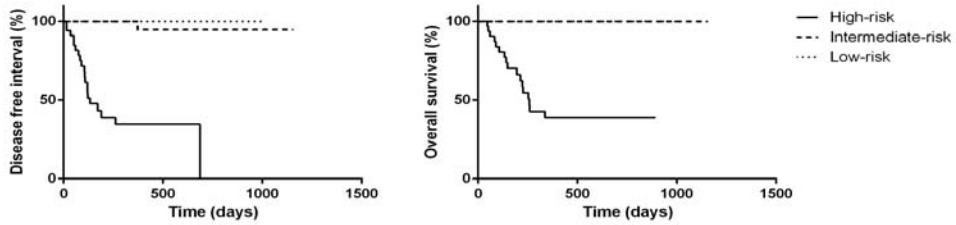

Figure 1. Graphical representation of the disease-free interval (left) and overall survival (right) of 89 dogs with mast cell tumours, according to the risk of recurrence and tumour dissemination. There is a difference in disease-free interval and overall survival, compared to the high risk group, since only this group reached the medians of 134 and 258 days, respectively (P <0.0001).

Significant differences in DFI and OS were observed only within the high-risk group. A DFI of 686, 107 and 109 days was assigned for groups A1, A2 and A3, respectively (p = 0,04), while the median OS was not reached for group A1, and it was significantly higher when compared to groups A2 (OS = 148 days, p = 0,003) and A3 (OS = 213 days, p = 0,0008). There was no differenc in DFI and OS between groups A2 and A3, as shown in figure 2. In the other groups the median was not reached, and no difference was observed, as only one out of 56 patients (1.8%) developed tumour recurrence at 372 days, but with no MCT related-deaths reported at the conclusion of the study.

Adverse side effects were mostly mild and self-limiting and are shown in table 3.

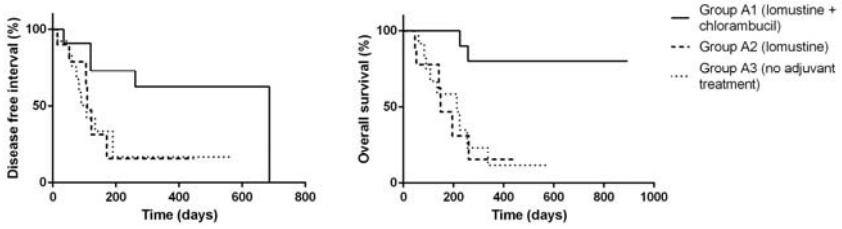

Figure 2. Graphical representation of disease-free interval (left) and overall survival (right) of 34 dogs with mast cell tumours at high risk of recurrence or metastasis, according to the use of adjuvant chemotherapy. A DFI of 686, 107 and 109 days was found for groups A1 (n = 11), A2 (n = 10) and A3 (n = 13), respectively (p = 0,04), while the median OS was not reached for group A1, and it was significantly higher when compared to groups A2 (OS = 148 days, p = 0,003) and A3 (OS = 213 days, p = 0,0008). There was no difference in DFI and OS between groups A2 and A3.

Table 3. Adverse effects to chemotherapy with lomustine, vinblastine and chlorambucil according to Veterinary Cooperative Oncology Group – Common Terminology Criteria for Adverse Events (Common..., 2011)

	Grade 1	Grade 2	Grade 3
LOMUSTINE (GROUPS A 1 AND A2; n = 21)			
Gastrointestinal toxicity	2	-	-
Neutropenia	9	6	2
Hepatic toxicity	10	2	-
VINBLASTINE (GROUP B1; n = 8)			
Gastrointestinal toxicity	-	-	-
Neutropenia	2	1	-
Hepatic toxicity	2	-	-
CHLORAMBUCIL (GROUP B2; n = 22)			
Hepatic toxicity	5	-	-

Gastrointestinal toxicity – grade 1 (less than 3 episodes of nausea/emesis in 24 hours, anorexia for 1-2 days, mild diarrhea), grade 2 (3-10 nausea/emesis episodes in 24 hours, anorexia for 3 days, moderate diarrhea), grade 3 (several episodes of nausea/emesis in 48 hours, anorexia for more than 3 days, severe diarrhea).
Neutropenia – grade 1 (>1500 neutrophils/μL), grade 2 (1000-1500 neutrophils/μL), grade 3 (500-999 neutrophils/μL).
Hepatic toxicity – grade 1 (asymptomatic but with increased alanine transaminase and alkaline phosphatase), grade 2 (symptomatic requiring medical intervention), grade 3 (synmptomatic requiring surgical intervention).

DISCUSSION

According to the European Consensus on MCT in dogs and cats, systemic therapy is considered an appropriate treatment for patients at high risk of metastasis (Blackwood *et al*., 2012). The prognostic factors for canine MCT used in this study are well-estabilished, therefore there was no intent to validate it in this study. A clear difference in outcome was found between the studied groups, with a worse disease control on high-risk patients compared to the intermediate-to-low risk groups patients. It must be considered that the intermediate risk criteria, used in this study, included prognostic factors with fewer evidence in literature (London and Thamm, 2013).

Traditionally, a higher risk of recurrence is suspected for canine MCT with histological evidence of incomplete or narrow surgical margins. However, this finding was not noticed in this study, and was also not observed in the study by Miller *et al*. (2014). In a study conducted by Michels *et al*. (2002), the likelihood of recurrence in MCT with evidence of incomplete surgical margins was not higher than those with clean margins. This may be the result of failure in the assessment of the surgical margins, or even by reduced importance of the extent of the surgery in the local control of canine MCT. But once tumour recurrence is associated with a poor outcome, with 86-100% of mortality rate, every effort should be made to prevent this (Bostock, 1973; Patnaik *et al*., 1984). Therefore, a revision surgery was performed, whenever possible, in all patients where clean margins could be achieved, in the abscence of features related to a higher-risk of recurrence or metastais, in order to reduce the risk of recurrence, downgrading them to the low-risk group, and preventing the inadvertent usage of systemic therapies. Considering the poor prognosis described for tumour recurrence, difficulty of a revision surgery in some cases and unavailability of radiotherapy, in this study, patients with incomplete or narrow surgical margins, without revision surgeries (intermediate-risk group), were also treated with systemic chemotherapy, but with a less immunosuppressive protocol, based on administration of vinblastine and or chlorambucil.

Once the mitotic count is a wll established prognostic factor there was no patient in the intermediate-to-low-risk groups with a mitotic count higher than five in 10 high power fields (Romansik *et al*., 2007). Nevertheless, a Ki-67 index higher than 1.8% was confirmed in 94,3% of all cases, and in 91,1% of the intermediate-to-low risk patients, and this may be related to a

low specificity of this prognostic factor, when compared to mitotic count, as described by van Lelyveld *et al*. (2015). Although internal tandem duplications in the c-*kit* oncogene (exon 11) were not prevalent in this study, these were present only in patients at a higher-risk of recurrence or metastasis, with concomitant negative prognostic factors, as reported by Zemke *et al*. (2002) and Webster *et al*. (2006).

No intermediate to low risk patient experienced MCT related death; however, in the high risk group, there was a high probability of death in patients presenting signs of disease progression (metastatic disease in skin or lymph nodes). Although some patients were followed for a short period of time, they assisted in the composition of the clinical picture, according to the risk of recurrence or metastasis.

Despite the many proposed drugs for chemotherapy in dogs with MCT, their use remains empirical (Dobson and Scase, 2007; London and Thamm, 2013) and the combination of vinblastine and prednisone appears to have become the protocol of choice (Dobson and Scase, 2007; Webster *et al*., 2008). In the study conducted by Thamm *et al*. (2006), 61 high-risk patients (intermediate to high grade MCT, located in mucocutaneous junctions or with regional lymph node involvement), received adjuvant treatment with intravenous vinblastine at a dosage of 2 mg/m^2 every seven or 14 days, combined with prednisone, resulting in a median survival of 1374 days for grade 3 MCT, whereas all patients with grade 2 MCT reached the survival of three years. Nevertheless, singularities of such population might have influenced such results and a poor outcome is usually seen in patients with a correct diagnosis of metastatic disease, meaning overt metastasis in lymph nodes (Weishaar *et al*., 2014), secondary skin nodules (satellite metastasis) and confirmed visceral involvement.

Other chemotherapeutic agents were also evaluated for treatment of canine MCT. In a study conducted by Taylor *et al*. (2009), dogs presenting with unresectable MCT were submitted to treatment with prednisolone and low-dose chlorambucil, with measurable clinical response in 38%, with no toxicity reported. Lomustine was also used in the neoadjuvant setting and proved effective, with measurable response in eight of the 19 treated dogs (42%) (Rassnick *et al*., 1999). Multi-agent protocols have shown promising results and association of vinblastine, cylcophosphamide and prednisone resulted in measurable responses in seven out of 11 dogs with cutaneous MCT (Camps-Palau *et al*., 2007). In the adjuvant setting, Cooper *et al*. (2009), treated 20 high-risk patients with prednisone, along with vinblastine (2mg/m^2, intravenously) and lomustine (27-90mg/m^2, orally), alternating every two weeks, and achieved a DFI and OS of 35 and 48 weeks, respectively.

However, despite the safety and remarkable effectiveness of lomustine and chlorambucil in the management of measurable disease (Rassnick *et al*., 1999; Taylor *et al*., 2009), this is the first study, involving patients adjuvantly treated with lomustine followed by chlorambucil (A1), lomustine (A2), vimblastine followed by chlorambucil (B1) and chlorambucil (B2), with control groups with similiar prognostic factors.

In this study, adjuvant lomustine was useful for control of canine MCT of high-risk of recurrence or metastasis, but only when sequentially associated to chlorambucil (group A1), which might be related to the small number of cases treated only with lomustine (group A2). Combination of lomustine with an eight-week chlorambucil protocol significantly improved DFI and OS of these patients. Further studies, with larger number of cases are needed to prove efficacy of this procol sequentially or concomitant to other chemotherapeutic agents.

Of 40 intermediate-risk patients (38 presenting with incomplete or narrow surgical margins), 30 received treatment with vinblastine and / or chlorambucil, in combination with prednisone, resulting in effective control of the disease in 97.5% of patients, and only one developed tumour relapse, with metastatic disease to the regional lymph node and satellite nodules on skin. This result is similar to findings obtained from patients presenting a similar fashion that were treated with radiotherapy or chemotherapy in the adjuvant setting (Al-Sarraf *et al*., 1996; Thamm *et al*., 2006; Cooper *et al*., 2009), and it is not different from the result achieved in the intermediate-risk control group (group B3) and low-risk (group C), without any divergence on DFI and OS. Systemic adjuvant treatment might

be unnecessary for patients with low-to-intermediate grade MCT, as they were defined in this study, even in the abscence of clean surgical margins, as also demonstrated by Smith *et al*. (2015). The classification according to the risk of recurrence or metastasis used in this study is not ideal and an improved one is necessary for a more accurate decision-making process about the use of adjuvant therapies.

The development of "de novo" MCT seem to occur sporadically (7.5%) in patients at intermediate risk, without any occurrence reported in those at low risk, however, as suggested by Davies *et al*. (2004), they are not associated with a worse outcome. Further studies with larger numbers of cases are therefore needed to define the need for adjuvant treatment in intermediate-risk patients.

The authors recognize the lack of randomization of this study, since chemotherapy was not performed in some cases and rescue therapies were attempted for some patients. Nevertheless, treatment could not be ethically denied for patients who might benefit from it, specially high-risk patients, with a clear indication for adjuvant chemotherapy.

Side effects were commonly associated with chemotherapy using lomustine or vinblastine, in association with prednisone. The myelosuppressive and hepatotoxic effects of lomustine in dogs are well recognized (Gustafon and Page, 2013) and this protocol was more toxic than the one with vinblastine. However, as reported by Cooper *et al*. (2009), despite the side effects ocurring in 54% of patients in a protocol alternating vinblastine and lomustine, these drugs were well tolerated by patients in this study. Thamm *et al*. (1999) reported side effects related to vinblastine in 20% of patients with MCT treated in the adjuvant setting, and as observed in this study, the side effects were, in general, mild and without any apparent decrease in the patient´s quality of life. Chlorambucil was definitely the safest protocol and abnormalities in ALT and ALP were probably related to glucocorticoids, nevertheless, long-term toxicities were not evaluated.

In dogs, most chemotherapeutic agents are calculated on the basis of body surface area, which is better correlated to physiologic parameters and heat loss, that may influence the pharmakinectics of drugs. Nevertheless, this dosing method provides a non-linear distribution of doses and may not be appropriate for all drugs. A greater toxicity is reported for melphalan, cisplatin, carboplatin and doxorubicin, in small dogs, whenever the dose is calculated on the basis of body surface area (Price and Frazier, 1998). For the majority of drugs, there is still lack of criteria for the most appropriate method for stating dosage in dogs and toxicity for the drugs used in this study, including prednisone, calculated on the basis of body surface area, may be different, according to the dog size or basal metabolism and new studies are necessary to show such differences.

CONCLUSION

Multi-agent therapy seems more effective for the adjunctive treatment of high-risk MCT, classified as grade 3 Patnaik, but also for those of intermediate grade, in the existence of lymph node or distant metastasis and / or high growth fraction estimated by the mitotic count. Sequential use of lomustine and chlorambucil, with concomitant glucocorticoids, was proven useful for those patients and it may extend survival and prevent tumour recurrence or metastasis. Patients at intermediate risk, similarly to those of low risk, may not require adjuvant treatments, even in the absence of free surgical margins.Vinblastine and/or chlorambucil did not prove advantageous for patients in this group. However, given the poor prognosis associated with canine MCT after recurrence, a large and careful evaluation of prognostic factors is necessary to rule out a higher risk of recurrence and tumour dissemination.

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