Treatment of multicentric or cranial mediastinal high-grade T-cell lymphoma in dogs with a first-line CCNU-L(-chlorambucil)-CHOP protocol

Eerstelijnsbehandeling met CCNU-L(-chloorambucil)-CHOP van honden met een hooggradig multicentrisch of mediastinaal T-cellymfoom

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ABSTRACT

This retrospective study determined disease free survival (DFS) and progression free survival (PFS) in chemo-naïve dogs with multicentric or cranial mediastinal high-grade T-cell lymphoma, treated with a first-line CCNU-L(-chlorambucil)-CHOP protocol. Of thirteen dogs with multicentric lymphoma, 92.3% achieved a complete remission (CR), and the median DFS and PFS was 317 and 256 days, respectively. Three dogs had cranial mediastinal lymphoma, and achieved a CR with a median DFS and PFS of 978 and 1007 days, respectively. The one- and two-year DFS/PFS probability estimate for dogs with multicentric lymphoma was 0.50/0.46 and 0.42/0.38, respectively, for dogs with cranial mediastinal lymphoma 0.67/0.67. Neutropenia and thrombocytopenia were reported in 52.9% and 50% of the dogs, respectively, while 56.3% experienced grade 1-4 nephrotoxicity, hypothesized to be lomustine-induced. It was concluded that, compared to historical data, the currently described first-line CCNU-L(-chlorambucil)-CHOP protocol could benefit the survival of dogs with multicentric or cranial mediastinal high-grade T-cell lymphoma.

SAMENVATTING

In dit retrospectieve onderzoek werd de ziektevrije- en progressievrije overleving bepaald van chemotherapie-naïeve honden met een hooggradig multicentrisch of mediastinaal T-cellymfoom, behandeld met een eerstelijns-CCNU-L(-chloorambucil)-CHOP-protocol. Van de dertien honden met een multicentrisch lymfoom vertoonde 92,3% een volledige remissie en de mediane ziektevrije- en progressievrije periode was respectievelijk 317 en 256 dagen. Drie honden hadden een mediastinaal lymfoom en vertoonden allemaal een volledige remissie met een mediane ziektevrije- en progressievrije periode van respectievelijk 978 en 1007 dagen. De één- en tweejarige ziektevrije/progressievrije overlevingskans voor honden met de multicentrische vorm was respectievelijk 0,50/0,46 en 0,42/0,38, voor honden met de mediastinale vorm 0,67/0,67. Neutropenie werd gevonden bij 52,9% van de honden, trombocytopenie bij 50% en 56,3% vertoonde een waarschijnlijk door CCNU veroorzaakte nefrotoxiciteit. De conclusie van het onderzoek is dat eerstelijnsbehandeling met CCNU-L(-chloorambucil)-CHOP een positief effect lijkt te hebben op de overlevingstijd van honden met een hooggradig multicentrisch of mediastinaal T-cellymfoom.

INTRODUCTION

T-cell lymphoma in dogs has been reported to occur with an incidence rate of 10-38% of all lymphoma subtypes (Teske et al., 1994a; Dobson et al., 2001; Fournel-Fleury et al., 2002; Rebhun et al., 2011). Although the WHO-classification scheme recognizes many distinct forms of T-cell lymphoma, only a few of these forms are commonly encountered in dogs. Peripheral T-cell lymphoma, not otherwise specified (NOS) and precursor T-cell lymphoblastic lymphoma are the two most common forms of high-grade T-cell lymphoma. This group of lymphomas is characterized by an aggressive clinical behavior with a poor

prognosis and a short-term response to traditional chemotherapy protocols (Teske et al., 1994b; Kiupel et al., 1999; Dobson et al., 2001; Ponce et al., 2004; Ponce et al., 2010; Valli et al., 2011; Valli et al., 2013). Four anatomical forms of high-grade T-cell lymphoma have been described: multicentric, alimentary, extra-nodal, and cranial mediastinal arising from the thymus and predominantly comprised of highly malignant T-lymphocytes (Owen, 1980). In comparison, T-zone lymphoma is a low-grade lymphoma with an indolent disease course and a good to excellent prognosis, either without treatment or treated with a combination of prednisolone and chlorambucil (Valli et al., 2013; Frantz et al., 2013). The validity of dividing T-cell lymphomas in dogs clinically into only two categories, i.e. high- and low-grade, was further confirmed in a molecular study (Frantz et al., 2013). Irrespective of the immunophenotype, CHOP-based protocols (cyclophosphamide, doxorubicin = hydroxydaunorubicin, vincristine = Oncovin®, and prednisolone) remain the standard of care for the treatment of high-grade lymphoma in dogs (Rebhun et al., 2011). Historical results of disease free survival (DFS) and progression free survival (PFS) in dogs with high-grade T-cell lymphoma, treated with CHOP-based protocols, range from 52 to 200 days, and are generally shorter compared to B-cell lymphoma (Vail et al., 1996; Ruslander et al., 1997; Chun et al., 2000; Ponce et al., 2003; Simon et al., 2006; Rebhun et al., 2011). Other treatment protocols, like L-MOPP (L-asparaginase, mechlorethamin, vincristine = Oncovin®, procarbazine, and prednisolone), did not bring a substantial improvement in survival outcome (Brodsky et al., 2009). Lomustine, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU), is an oral alkylating agent of the nitrosourea subclass. In dogs, CCNU is typically reserved for the treatment of relapsed or refractory lymphoma, either as a single agent or as part of a multidrug protocol (Moore et al., 1999; Saba et al., 2007; Flory et al., 2008; Saba et al., 2009). Up to now, information on the additive value of CCNU within a first-line treatment protocol for canine high-grade T-cell lymphoma has been limited (Morrison-Collister et al., 2003; Sauerbrey et al., 2007; Rassnick et al., 2009; Rassnick et al., 2010).

The aim of this retrospective study was to determine DFS and PFS in dogs with high-grade multicentric or cranial mediastinal T-cell lymphoma, treated with a first-line CCNU-L-CHOP protocol (CCNU, L-asparaginase, cyclophosphamide, doxorubicin = hydroxydaunorubicin, vincristine = Oncovin®, and prednisolone), with or without a single high dose of chlorambucil in the induction phase, CCNU-L(-chlorambucil)-CHOP, and investigate the toxicity of this protocol. The hypothesis was that the addition of chlorambucil in the induction phase, and CCNU in both the induction and maintenance phase of a first-line L-CHOP protocol would improve DFS and PFS compared to historical data of L-CHOP-based protocols for dogs with high-grade T-cell lymphoma.

MATERIAL AND METHODS

Study population

Medical records from de Ottenhorst, Veterinary Oncology Referral Centre, Terneuzen, the Netherlands (January 2003 – August 2015) were retrospectively analyzed. The dogs that were included, had a cytologically or histologically confirmed and immunophenotyped high-grade multicentric or cranial mediastinal T-cell lymphoma, with an intention to treat with CCNU-L(-chlorambucil)-CHOP as first-line therapy. Dogs were excluded if they were diagnosed with low-grade, indolent (T-zone) and other extra-nodal forms of lymphoma, or concurrent other diseases that severely limited life expectancy or prevented the use of the intended oncolytic drugs. Concurrent use of prednisolone for a maximum of three weeks was allowed for dogs entering the study, provided the diagnosis of high-grade T-cell lymphoma had already been made before the start of corticosteroid therapy and could be re-evaluated. Concurrent or previous use of other drugs was allowed, although the dogs had to be chemo-naïve.

Diagnosis and staging

Diagnosis was made by cytology and/or histology, combined with immunocytochemistry or immunohistochemistry typically using antibodies against CD3 (T-cell marker) and the CD79a (B-cell marker) (Dako, Glostrup, Denmark). Cytology samples were classified according to the updated Kiel classification and histology samples based on the WHO classification (Wright, 1987; Teske et al., 1994b; Fournel-Fleury et al., 1997; Ponce et al., 2010; Valli et al., 2011). Staging and substaging was performed, based on the World Health Organization TNM Classification of Tumors of Domestic Animals (Owen, 1980). Pretreatment assessment included a complete blood cell count (CBC), a complete serum biochemistry profile, and in case clinically indicated, thoracic radiographs and abdominal ultrasound.

Treatment protocol

Dogs were treated with a CCNU-L-CHOP protocol, with the intention to include a single dose of chlorambucil of 1.4 mg kg^{-1} in week 7 of the protocol, and the first CCNU administration planned in the induction phase of the protocol between week 5-11 (Table 1). Prior to this first dose of CCNU, the dogs were required to have a normal kidney function, defined as serum urea and creatinine levels within the reference range and no abnormalities on urinalysis (specific gravity plus dipstick, urine protein to creatinine ratio when indicated), an ALT ≤ 4x the upper reference value used in the Ottenhorst centre, a neutrophil count ≥ $3x10^{9-L}$ and a thrombocyte count ≥ $150x10^{9-L}$. The CCNU dose was planned between

Table 1. CCNU-L-chlorambucil-CHOP protocol. Subsequent to each CCNU dose in the maintenance phase, the next vincristine dose was administered after three weeks.

Drug	Dose	Induction phase											Maintenance phase (5 cycles)			
		Week											Week			
		0	1	2	3	4	5	6	7	8	9	11	14	15	16	18
L-asparaginase	400 IU kg^{-1} IM	•														
Vincristine	0.5-0.7 mg m^{-2} IV		•		•			•		•			•			
Cyclo-phosphamide	250 mg m^{-2} over 2 consecutive days PO			•										•		
Doxorubicin	30 mg m^{-2} IV					•					•				•	
CCNU	60-90 mg m^{-2} PO						1 dose									•
Chlorambucil	1.4 mg kg^{-1} PO								•							
Prednisolone	mg kg^{-1} BID PO		1	0.75	0.5	0.5 e.o.d.*							0.5 e.o.d.*			

*Every other day

60-90 mg m^{-2}, with the dose adjusted to the nearest dose that could be administered using available whole capsules of 10, 15 and 40 mg of CCNU.

Since a CBC was not routinely performed at the expected neutrophil nadir five to seven days after the administration of CCNU, but only prior to the next chemotherapy dose, a prophylactic antibiotic therapy (amoxicillin-clavulanate or quinolones) was commenced 48 hours after CCNU administration and continued for a total of eight days.

Assessment of adverse events

Treatment-associated adverse events were graded according to the VCOG-CTCAE v1.1 grading scheme (VCOG-CTCAE, 2011). A physical examination and CBC were performed before each administration of chemotherapy, and between treatments when indicated based on clinical signs. Prior to each CCNU administration, renal function was assessed by measuring a serum creatinine level and performing a urinalysis. Proteinuria was quantified using a urine protein to creatinine ratio when the dipstick test was positive for protein. Liver enzymes were not routinely measured, but only based on clinical suspicion of hepatotoxicity, as a general not well-being, decreased appetite, vomiting or diarrhea, weight loss, and lethargy. Gastrointestinal toxicity was scored on the basis of patient history.

Assessment of response

Since most of the dogs were treated before 2010, the VCOG consensus document, describing the evaluation criteria for the treatment response of peripheral nodal lymphoma in dogs, could not be followed (Vail et al., 2010). The treatment response was categorized as follows: complete remission (CR), complete disappearance of all measurable disease; partial remission (PR), > 50% reduction but < 100% reduction in volume of all measurable disease; stable disease (SD), < 50% reduction in volume of all measurable disease and < 25% increase in volume; progressive disease (PD), > 25% increase in volume of all measurable disease.

In case of multicentric lymphoma, the remission status was determined at each visit, based on the results of physical examination and calliper measurement of lymph node sizes. Treatment response for cranial mediastinal lymphoma was monitored through physical examination, thoracic radiography and/or ultrasonography at week 3 or 4, and repeated at week 8 or 9. Further follow-up assessment for cranial mediastinal lymphoma was performed as indicated by clinical signs or owner's preferences. After completing the treatment, a first follow-up visit was planned one month later; thereafter, every three months.

Study endpoints

The endpoints of the study were disease free survival (DFS) and progression free survival (PFS). DFS was defined as the time from achieving complete remission until the time of first relapse, or tumor or therapy related death. PFS was defined as the time between treatment initiation and tumor progression, or tumor- or therapy-related death.

Statistical analysis

The Kaplan-Meier product limit method was used for calculating DFS and PFS (IBM SPSS

Statistics V21.0 software package). The log-rank test was performed to demonstrate significant differences between patient subgroups, with the level of significance set at a P-value < 0.05. By identifying specific patient subgroups, further assessments were made of the prognostic significance of prior corticosteroid therapy, stage of the disease, presence of hypercalcemia, whether or not dogs received chlorambucil, and the moment of CCNU administration. For survival analysis, the dogs without progression of their lymphoma and alive at the end of the study, or that had died from causes not related to lymphoma or therapy, were censored. The censoring date was defined as the last date, on which the progression status was adequately assessed.

RESULTS

Patient characteristics

The files of 65 dogs diagnosed with T-cell lymphoma were retrospectively analysed. Twenty-eight dogs were diagnosed with low-grade lymphomas, of which 13 cutaneous, and 37 dogs with high-grade lymphoma. Seven dogs were not treated based on owners' decision, 14 were treated with different protocols. Sixteen dogs met the inclusion criteria. The patient characteristics are summarized in Table 2. The most common breeds were the Labrador retriever (n = 3) and Dogue de Bordeaux (n = 3). Thirteen dogs had multicentric, and three cranial mediastinal lymphoma. Thirteen dogs were diagnosed with a high-grade T-cell lymphoma by means of a combination of cytology and immunocytochemistry, whereas the diagnosis of three other dogs was based on histopathology and immunohistochemistry. Of those three, two were classified as an anaplastic large T-cell lymphoma, one as peripheral T-cell lymphoma NOS. Fourteen dogs were reviewed by one pathologist, and of two dogs, cytology and histology were performed in two different diagnostic pathology labs.

Seven dogs presented with hypercalcemia: six with multicentric and one with cranial mediastinal lymphoma.

Two dogs with multicentric and one dog with cranial mediastinal lymphoma had been treated with prednisolone with a median duration of 13 days (range: 9-17) prior to the initiation of chemotherapy.

Treatment analysis

Eight dogs completed the protocol: six with multicentric and two with a cranial mediastinal lymphoma. In five dogs, the therapy was prematurely stopped because of tumor progression, in one dog because of a severe increase in serum creatinine concentration, and two dogs died because of septicemia. The median duration of the CCNU-L(-chlorambucil)-CHOP protocol in those dogs that completed the treatment was 289 days (range: 224-514), with a median number of 5 CCNU doses delivered (range: 4-6). The median cumulative dose of CCNU in this group of dogs was 405 mg m^{-2} (range:

Table 2. Patient characteristics. F = female; Fn = female neutered; M = male; Mn = male neutered; CR = complete remission; DFS = disease free survival; PFS = progression free survival; N/A = not applicable; TR = tumor related; RF = renal failure.

Breed	Age (years)	Gender	Stage and substage	Hyper-Ca	Chlor-ambucil	CCNU in induction	Res-ponse	DFS	PFS	Cause of death
Multicentric lymphoma (n = 13)										
Airedale terrier	10.4	M	III a	-	+	+	CR	160	201	TR
Boxer	6.5	Fn	III a	+	+	-	CR	194	199	Septicemia (neutropenic)
Bullterrier	6.1	F	III a	-	-	+	CR	242	256	TR
Dogue de Bordeaux	4.0	Fn	III a	+	+	+	CR	848	882	Lone atrial fibrillation
Mixed-breed	5.8	Mn	III a	-	+	-	CR	225	243	TR
Australian shepherd	5.8	Fn	III b	-	-	+	CR	1365	1377	Euthanasia on owners request
Dogue de Bordeaux	4.0	Fn	III b	+	+	+	CR	867	883	Hemangiosarcoma
White Swiss shepherd	8.2	M	III b	+	-	+	CR	24	45	TR
Dogue de Bordeaux	2.5	M	IV a	-	+	+	CR	395	399	Accident
X English Stafford	10.1	M	V a	-	-	+	PR	N/A	87	TR
Labrador retriever	1.3	Fn	V b	+	-	+	CR	104	116	TR
Labrador retriever	6.5	Mn	V b	-	+	+	CR	887	891	TR
Scottish collie	4.3	Fn	V b	-	-	+	CR	392	401	RF
Cranial mediastinal lymphoma (n = 3)										
Mixed-breed	11.5	Mn	V b	-	+	-	CR	200	227	Septicemia (non-neutropenic)
Golden retriever	1.7	M	V b	+	+	+	CR	1578	1596	RF
Labrador retriever	5.5	Fn	V b	-	-	+	CR	978	1007	RF

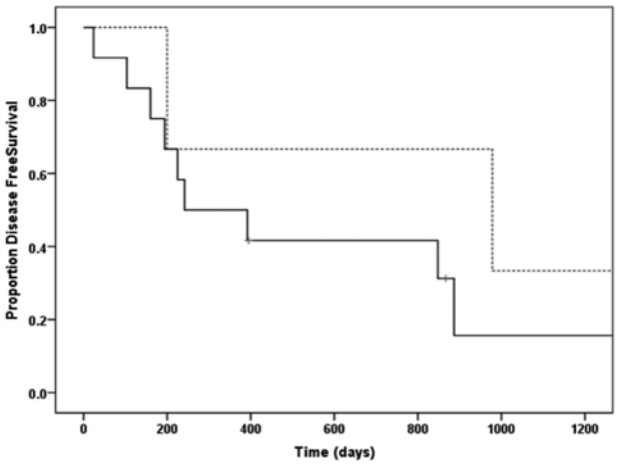

Figure 1. Kaplan-Meier curves for disease fee survival (DFS) per anatomical location. The one- and two-year DFS probability estimate for dogs with multicentric lymphoma was 0.50 and 0.42, respectively, for dogs with cranial mediastinal lymphoma 0.67 and 0.67, respectively.
Multicentric ————————
Cranial mediastinal …… .

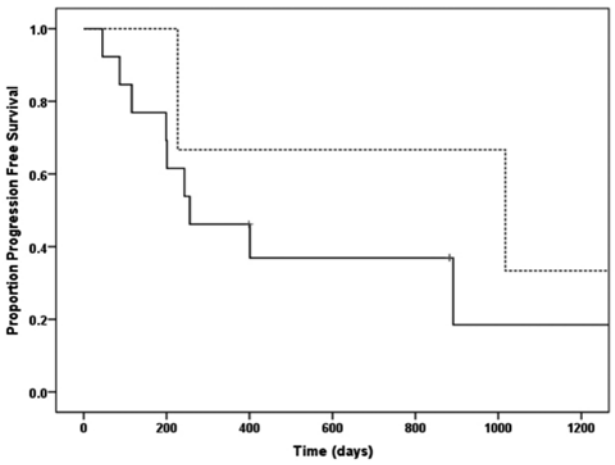

Figure 2. Kaplan-Meier curves for progression fee survival (PFS) per anatomical location. The one- and two-year PFS probability estimate for dogs with multicentric lymphoma was 0.46 and 0.38, respectively, for dogs with cranial mediastinal lymphoma 0.67 and 0.67, respectively.
Multicentric ————————
Cranial mediastinal …… .

320-480 mg m^{-2}), with a median single-dose of 80 mg m^{-2} (range: 70-90 mg m^{-2}).

Nine of the sixteen dogs received a single dose of 1.4 mg kg^{-1} chlorambucil: seven of the thirteen dogs with multicentric lymphoma during week 7, and two of the three dogs with cranial mediastinal lymphoma during week 9 (Table 2). Dogs that did not receive chlorambucil in the induction phase, had already received the first dose of CCNU before week 7, based on the clinician's judgement. The first dose of CCNU was delivered in 13 dogs between week 5-11. Two dogs with multicentric and one dog with cranial mediastinal lymphoma received their first dose of CCNU only in the maintenance phase of the protocol between week 14-16 (Table 2).

Response to treatment

Of the 13 dogs with multicentric lymphoma, 12 (92.3%) achieved a CR and one a PR, between week 0-5. The median DFS in this group was 317 days (range: 24-1365), and the median PFS 256 days (range: 45-1377). The median DFS and PFS for the dogs that received the first dose of CCNU during the induction phase of the protocol was 394 and 399 days, and for the two dogs that received the first dose of CCNU in the maintenance phase 210 and 221 days, respectively. The median DFS and PFS for the dogs that received chlorambucil was 395 and 399 days, and for the dogs that did not, 242 and 186 days, respectively. The individual survival times and cause of death for the dogs with multicentric lymphoma are shown in Table 2. The Kaplan-Meier survival plot for DFS and PFS of the dogs with multicentric lymphoma is outlined in Figures 1 and 2.

All three dogs with cranial mediastinal lymphoma achieved a CR within 18, 27, and 29 days, confirmed by thoracic radiographs and/or ultrasonography, respectively. The median DFS and PFS in this group was 978 and 1007 days, respectively. The median DFS and PFS for the two dogs that received the first dose of CCNU before D80 was 1278 and 1301 days. DFS and PFS for the dog that received the first dose of CCNU at D105 was 200 and 227 days, respectively. DFS for the two dogs that received chlorambucil was 200 and 1578 days, respectively, and for the dog that did not, 978 days. PFS for the two dogs that received chlorambucil was 227 and 1596 days, respectively, and for the dog that did not, 1007 days. The individual survival times and cause of death for the dogs with cranial mediastinal lymphoma are shown in Table 2. The Kaplan-Meier survival plot for DFS and PFS of dogs with cranial mediastinal lymphoma is outlined in Figures 1 and 2.

DFS and PFS did not differ significantly for dogs with hypercalcemia versus normocalcemia (P = 0.842 and P = 0.480, respectively), pretreatment with prednisolone versus no pretreatment (P = 0.792 and P = 0.361, respectively), with or without a single dose of chlorambucil in the induction phase (P = 0.543 and P = 0.269, respectively), with the first dose of CCNU in the induction or maintenance phase (P = 0.059 and P = 0.091, respectively), stage 3, 4 or 5 of the disease (P = 0.811 and P = 0.944, respectively), substage a or b (P = 0.366 and P = 0.426, respectively), and anatomic location (P = 0.231 and P = 0.254, respectively).

Adverse events

The distribution of the adverse events is shown in Table 3. Neutropenia and thrombocytopenia was reported in 52.9% and 50.0% of the dogs, respectively, and anemia in 68.8%. In most of the cases, these bone marrow related adverse events were defined as grade

1 or 2. However, one dog died because of neutropenia related septicemia in combination with diarrhea, ten days after doxorubicin and eight weeks after CCNU administration.

Nine dogs (56.3%) experienced signs of nephrotoxicity during or after the treatment period. The first laboratory sign in five of them was proteinuria, in three an elevation of the serum creatinine concentration, and one dog presented both proteinuria and creatinine elevation at the first diagnosis of kidney damage. The first signs of nephrotoxicity were noted after a median and mean of 184 and 296 days of treatment (range: 58-876), respectively, and after a median and mean of 4 CCNU doses (range: 1-6). In five dogs, nephrotoxicity was observed during the protocol. In four dogs, the first signs of renal disease appeared after finishing the treatment with a median and mean of 184 and 194 days after the last chemotherapy dose. Kidney parameters, being serum urea, creatinine, and phosphate concentration, and urine specific gravity and protein/creatinine ratio, failed to improve in any of these dogs during the follow-up period, deteriorated slowly, and renal failure was the cause of death in one dog with multicentric and two dogs with cranial mediastinal lymphoma (Table 2). Four of the six hypercalcemic dogs and five of the ten normocalcemic dogs developed renal disease.

Eight dogs (50.0%) had short delays in the administration of various chemotherapeutic drugs with a median number of seven days (range: 4-12). The most frequent cause was neutropenia ≥ grade-1. Only in one dog, hepatotoxicity (grade 3) was noted.

Table 3. Distribution of the most common adverse events according to the VCOG-CTCAE v1.1 grading scheme (2011).

	CCNU-L-CHOP (n = 16)
Neutropenia	9 (52.9%)
Grade 1	7 (77.7%)
Grade 2	1 (11.5%)
Grade 5	1 (11.5%)
Thrombocytopenia	8 (50%)
Grade 1	4 (50%)
Grade 2	3 (37.5%)
Grade 3	-
Grade 4	1 (12.5%)
Anemia	11 (68.8%)
Grade 1	9 (81.8%)
Grade 2	2 (18.2%)
Renal toxicity	9 (56.3%)
Increased creatinine	7 (77.7%)
Grade 1	4 (57.1%)
Grade 2	1 (14.3%)
Grade 3	1 (14.3%)
Grade 4	1 (14.3%)
Proteinuria	6 (32%)*
Grade 1	3 (75%)
Grade 2	0 (0%)
Grade 3	1 (25%)
Hepatotoxicity	1 (5%)
Grade 3	1

*In two dogs, proteinuria was only determined by means of a heat precipitation test and could not be graded.

DISCUSSION

In humans, there is a broad range of different T-cell lymphomas that have unique characteristics and often warrant individualized diagnostic and therapeutic treatment strategies (Rizvi et al., 2006). In the dog however, the vast majority of T-cell lymphomas belong to the precursor T-cell lymphoblastic subtype and the peripheral T-cell lymphoma NOS, both high-grade lymphomas, and the mature T-cell or T-zone lymphoma, that is a low-grade lymphoma (Ponce et al., 2010; Valli et al., 2011). In addition to these nodal forms, there is the group of cutaneous epitheliotropic T-cell lymphomas.

In the current study, multicentric and cranial mediastinal high-grade T-cell subtypes, diagnosed on cytology or histology were included, and in the response and survival analysis separately evaluated. The validity of dividing T-cell lymphomas in the dog into only two categories, i. e. high-grade and low-grade, was confirmed in a molecular study (Frantz et al., 2013). Lymphoblastic T-cell lymphomas and peripheral T-cell lymphomas NOS were composed of a single molecular group (consistently named high-grade T-cell lymphoma), while low-grade T-zone lymphoma consisted of a distinct molecular group (consistently named low-grade T-cell lymphoma). In addition, this study showed that the two high-grade T-cell types had the same poor prognosis, while the low-grade T-cell lymphomas performed much better (Frantz et al., 2013). These findings were further confirmed by the study of Valli et al. in 2013. Epitheliotropic cutaneous and low-grade T-cell lymphomas were therefore excluded from the current study. Since a consensus on the use of the term high-grade lymphoma seems to be lacking, and in line with the previously mentioned studies, this name is used consistently in the current study to point out the aggressive biologic behavior of the lymphomas diagnosed on cytology as well as on histology.

Only three dogs had their diagnosis based on histology, while in 13 dogs, the diagnosis of lymphoma was based on cytology. In large series of lymphomas in dogs, cytology has been demonstrated to have a positive correlation with the WHO classification with respect to the previously mentioned three most commonly diagnosed subtypes of T-cell lymphoma (Teske et al., 1994a; Ponce et al., 2010; Jankowska et al., 2015). Transformation of low-grade lymphomas into high-grade has been reported in humans as well as in dogs, albeit only for the B-cell phenotype in the latter species (Comazzi et al., 2015). Even when well-advanced, T-zone lymphoma still has the small mature

appearing cell type and paucity of mitotic activity. As a result, it cannot easily be confused on cytology with a high-grade lymphoma (Valli et al., 2006). This makes it very unlikely that advanced T-zone lymphomas were not recognized in the current study on cytology, and could have biased the survival outcome.

It is difficult to compare survival data between studies. Differences in endpoints, definition of endpoints, censoring, classification, lack of immunophenotyping and selection of the inclusion and exclusion criteria make comparison unreliable. In the current study, PFS was selected as a second endpoint. Overall survival time, as opposed to PFS, is influenced by many factors, including the re-induction, rescue protocols used, and the owner's willingness to continue treatment. In the statistical analysis, many of the previous studies excluded dogs that did not achieve a CR. It is undeniable that the main treatment goal in dogs with lymphoma is complete remission and that achieving CR is a favorable prognostic factor. This is the reason why DFS was the first endpoint in the present study. However, if CR is not attainable, a PR or SD, preserving a good quality of life, is also an acceptable treatment endpoint, reflected in the PFS.

CCNU is frequently used in rescue protocols for dogs with B- and T-cell lymphoma (Moore et al., 1999; Saba et al., 2007; Flory et al., 2008; Saba et al., 2009). However, to determine the efficacy of the treatment of a specific tumor type with a specific drug, this drug has to be used in first-line therapy, since the outcome of the therapy of resistant or relapsed tumors can be associated with the induction of multi-drug resistance. Only four published studies incorporated CCNU in first-line treatment protocols for multicentric or gastrointestinal high-grade lymphoma in dogs, focused on the improvement of the survival outcome (Morrison-Collister et al., 2003; Sauerbrey et al., 2007; Rassnick et al., 2009; Rassnick et al., 2010). In two of these studies, CCNU was used in the consolidation phase of a first-line non-CHOP protocol (Morrison-Collister et al., 2003; Rassnick et al., 2009). One of these studies exclusively dealt with dogs with gastro-intestinal involvement of the lymphoma, 63% being of T-cell origin. The median survival time for the dogs with T-cell lymphoma was 22 days, so most of the dogs never reached the consolidation phase (Rassnick et al., 2009). In the other study, including dogs with multicentric lymphoma, 38% had the T-cell immunophenotype. CCNU was also incorporated in the consolidation phase, resulting in a median DFS of 168 days, but the results were unfortunately not stratified by immunophenotype (Morrison-Collister et al., 2003). Another study in dogs with multicentric lymphoma, not further graded and immunophenotyped, combined CCNU with prednisone as a first-line treatment, resulting in a 53% response rate with a median PFS of 39.5 days (Sauerbrey et al., 2007). In the study of Rassnick et al. (2010), dogs with multicentric T-cell and B-cell lymphoma were treated first-line with an L-CHOP-CCNU-MOPP protocol. CCNU was incorporated only twice during the treatment: once in the induction phase and once in the maintenance phase. The median DFS for dogs with multicentric T-cell lymphoma was 126 days, compared to 379 days for dogs with multicentric B-cell lymphoma. However, this difference was not significant. Overall, the outcome of these studies compares unfavorably to the median DFS and PFS reported in the current study. Moreover, the one- and two-year DFS- and PFS-rate in the current study corresponds to the historical results of B-cell lymphoma treatment with CHOP-based protocols (Keller et al., 1993; Garrett et al., 2002).

Since this was a retrospective study, in which the idea to incorporate chlorambucil and CCNU in a preexisting L-CHOP protocol arose more than ten years ago, and the moment of first delivery of these two drugs was based on the clinicians judgement, it turned out that not all dogs received chlorambucil. Additionally, there was a relatively wide variation in time the first CCNU dose was administered. The dogs that did not receive chlorambucil and the dogs that had CCNU administration postponed to the maintenance phase, seem to have a shorter DFS. However, this observation is limited because of the small number of patients, and hence the low statistical power.

One possible explanation for the survival benefit of dogs, treated with CCNU-L(-Chlorambucil)-CHOP for high-grade multicentric or cranial mediastinal T-cell lymphoma is the fact that it is an alkylating agent-rich protocol. Previous studies suggested that the inclusion of multiple alkylating agents within chemotherapy protocols to treat high-grade lymphoma in dogs results in the T-cell phenotype no longer being a negative prognostic factor (Morrison-Collister et al., 2003; Saba et al., 2009).

T-cell lymphomas seem to have a considerably higher number of chromosomal aberrations than B-cell lymphomas (Thomas et al., 2003). This genetic instability could allow T-cell lymphomas to develop methods to evade the cytotoxic effects of chemotherapeutic agents more easily than their B-cell counterparts (Goldie and Coldman, 1984). In one study, T-cell lymphomas actually showed a higher degree of intrinsic drug resistance than B-cell lymphomas (Zandvliet et al., 2015). On the other hand, it is known that alkylating agents are not a substrate of P-glycoprotein efflux mechanisms, and cross resistance is uncommon (Teicher et al., 1986; Borst 2012; Chakkath et al., 2014).

The incorporation of CCNU into CHOP-based protocols has been reported to cause an increase in the incidence of adverse events, with neutropenia, thrombocytopenia and hepatotoxicity being most commonly reported (Morrison-Collister et al., 2003; Kristal et al., 2009; Hosoya et al., 2009; Heading et al., 2011; Skorupski et al., 2011). In the current study, bone marrow toxicities were reversible and in most cases of low-grade. As a shortcoming of this study, mostly for practical or financial reasons, no CBC was

performed at the expected nadir of the neutrophil count, five to seven days after CCNU administration. Consequently, higher grade neutropenia's potentially could have been missed. In an attempt to prevent septicemia, a prophylactic antibiotic therapy was started from two till ten days after the CCNU. The selected antibiotic changed in the course of time from quinolone antibiotics to amoxicillin-clavulanate, based on the regulations on the use of antibiotics in companion animals in the Netherlands. One dog died as a result of non-CCNU related septicemia during an episode of diarrhea, ten days after doxorubicin administration, while the last dose of CCNU was delivered eight weeks before. In this dog, a CBC was performed by the referring veterinarian one week after doxorubicin and at that time, the neutrophil count was normal, but rapidly deteriorated in combination with diarrhea. In the group of the dogs with a cranial mediastinal lymphoma, one dog died one hour after developing acute high fever with a normal neutrophil count, 14 days after cyclophosphamide administration and eight weeks after CCNU.

The second most frequent adverse event was nephrotoxicity, that occurred in nine dogs (56.3%) of the study population. So far, renal toxicity has been reported to be a rare side effect of CCNU in both human and veterinary medicine (Saba et al., 2007; Brodsky et al., 2009; Kristal et al., 2009; Sahni et al., 2009). Although one human study reported 17 of 18 patients who received at least six courses of CCNU for treatment of brain tumors, and all of the nine patients who received more than ten courses of CCNU, to develop impaired renal function. Renal tissue, obtained by percutaneous biopsy in five patients and on post-mortem examination in two patients, demonstrated tubular atrophy, interstitial fibrosis and glomerular sclerosis. The remarkably high incidence of renal damage in this human patient group occurred without a phase of acute renal failure and in the absence of significant urinary abnormalities, while producing insidiously progressive interstitial renal lesions (Schacht et al., 1981). One veterinary study reported that 22.7% of dogs, treated with a CCNU-based protocol for a variety of neoplastic diseases, demonstrated renal toxicity (Bavcar et al., 2013). In general, in humans, nitrosoureas cause renal tubular cell damage by alkylating renal macromolecules like DNA, RNA and various enzymes, which in turn results in tubular atrophy, glomerular sclerosis and chronic tubulo-interstitial nephritis. This leads to a slowly progressive renal dysfunction and finally renal failure, starting either concurrently with the administration of the drug or even months following discontinuation of therapy (Schacht et al., 1981; Sahni et al., 2009). The same pattern seems to be present in the currently reported CCNU-L(-chlorambucil)-CHOP protocol. However, it cannot be excluded that the observed nephrotoxicity resulted from the combined use of CCNU with other oncolytic drugs and antibiotics, and is not related to the use of CCNU alone. Nevertheless, the number of dogs could be insufficient to make a definitive statement. Hypercalcemia prior to the initiation of a CCNU-based therapy did not appear to increase the risk of developing nephrotoxicity.

CCNU is known to be hepatotoxic. Acute injury appears to be localized in the large bile ducts and reflects inflammatory edema, bile stasis, and degeneration of epithelial cells, leading to pericholangitis, intrahepatic cholestasis, secondary hepatocyte injury, and in the long term biliary cirrhosis (Kretschmer et al., 1987). The most common hepatic histopathologic abnormalities in dogs, at the moment of clinical signs, are also inflammatory in nature, and include mild to moderate neutrophilic and lymphoplasmacytic periportal inflammation, fibrosis, and mild bile duct hyperplasia (Kristal et al., 2009). In one study comprising 179 dogs treated with CCNU, 11 dogs (6.1%) developed hepatic toxicity, following a median of 4 CCNU doses and a median cumulative dose of 350 mg m^{-2}. Median duration to detection of hepatic toxicity from the last dose of CCNU was 11 weeks (range 2-49 weeks). Clinical signs resolved in some dogs, but biochemical abnormalities and histopathologic lesions persisted 4-38 months from the time of diagnosis of liver disease. The majority of the dogs with CCNU-induced hepatotoxicity died from progressive liver disease (Kristal et al., 2009). These findings suggest that CCNU can cause delayed, cumulative dose-related, chronic hepatotoxicity that is irreversible and almost always fatal. High concentrations of toxic metabolites in the bile could be responsible for the hepatotoxic effects of CCNU (Kristal et al., 2009).

CCNU induced hepatotoxicity was of concern in the current study, but since only one case of hepatotoxicity (grade 3) was diagnosed, it did not appear to be a common adverse event, although the dogs were not routinely checked, and subclinical cases might have been undetected. Increases in serum liver enzymes were mostly mild and not associated with clinical signs and might theoretically also have resulted from the concomitant prednisolone administration. Although limited assessment of liver enzymes is a shortcoming of this retrospective study. The chance that clinically relevant hepatotoxicity was missed is considered low due to the absence of appropriate clinical signs. Also those dogs that lived long after treatment, did not show signs of hepatic disease. The authors hypothesize that, since all dogs were on an immunosuppressive dose of prednisolone, this could have prevented the CCNU related hepatotoxicity. Also based on the reported irreversible nature of the liver damage by CCNU with an almost one hundred percent fatal outcome, it is very unlikely that undetected CCNU-related hepatotoxicity occurred in this group of dogs. Despite this observed lack of CCNU induced hepatic failure in the current study, the effect of liver damage, as previously described, must not be underestimated when treating dogs with CCNU, and should be carefully monitored in prospective studies

(Kristal et al., 2009; Hosoya et al., 2009; Heading et al., 2011; Skorupski et al., 2011).

It is concluded that the currently described first-line CCNU-L(-chlorambucil)-CHOP protocol can benefit survival of dogs with high-grade T-cell lymphoma compared to historical results of L-CHOP-based protocols. Although the retrospective character of this study and the small number of dogs treated give rise to limitations, the current results warrant a prospective trial on the use of CCNU-L-chlorambucil-CHOP treatment as a first-line protocol for dogs with high-grade T-cell lymphoma.

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