Prognostic significance of clinical presentation, induction and rescue treatment in 42 cases of canine centroblastic diffuse large B-cell multicentric lymphoma in the UK

Owen Davies[1], Balazs Szladovits[2], Gerry Polton[3], Oliver A Garden[4], Chiara Leo[5], Ana Lara-Garcia[6]

[1]Highcroft Veterinary Referrals, 615 Wells Road, Whitchurch, Bristol. UK. BS14 9BE.

[2]Department of Pathobiology & Population Sciences, Royal Veterinary College, University of London, North Mymms, AL9 7TA, UK.

[3]North Downs Specialist Referrals, The Friesian Buildings 3 & 4, The Brewerstreet Dairy Business Park, Brewer Street, Bletchingley RH1 4QP, UK

[4]Department of Clinical Studies – Philadelphia, University of Pennsylvania, School of Veterinary Medicine, 3900 Spruce Street, Philadelphia, PA 19104, USA.

[5] Istituto Veterinario di Novara, Strada Provinciale, 9, 28060 Granozzo con Monticello NO, Italy.

[6]Queen Mother Hospital for Animals, Royal Veterinary College, University of London, North Mymms, AL9 7TA, UK.

Part of this data was presented in oral abstract form at the ECVIM-CA Congress, Lisbon, Portugal, September 2015.

Corresponding author: Owen Davies, Highcroft Veterinary Referrals, 615 Wells Road, Bristol. UK. BS14 9BE. Email address: owen.davies@highcroftvet.co.uk

Abstract :

Canine lymphoma is a heterogeneous group of diseases and many previous studies have evaluated the response of a mixed population of lymphoma cases to one specific treatment protocol. The aim of this retrospective study was to describe the outcome and prognostic factors in 42 cases of multicentric centroblastic diffuse large B-cell lymphoma treated with either a COP-type (35%) or CHOP (64%) induction chemotherapy. The objective response rate to induction therapy was 94%; entire dogs had a greater rate of complete versus partial remissions than neutered dogs (P=0.017). Median progression free survival for the first remission (PFS1) was 182 days; absence of anaemia at diagnosis (P=0.002) and pre-treatment neutrophil:lymphocyte ratio (NLR) below 9.44 (P=0.015) were independently predictive of longer PFS1. Fifty-eight percent of dogs received rescue protocols with an objective response rate of 81%; 31% of dogs received further rescue protocols (up to a total of 5) and the median number of protocols administered was 2. Median overall survival (OS) was 322 days, the 1-year survival rate was 38% and the 2-year survival rate was 9%. Lymphocyte:monocyte ratio (LMR) above 1.43 (P=0.031), NLR below 11.44 (P=0.009), the combination of induction and rescue therapy (P=0.030) and the total number of doxorubicin doses used (P=0.002) were independently predictive of longer OS. Use of a COP-type protocol induction compared to CHOP did not undermine OS providing doxorubicin was used as rescue therapy.

Keywords : COP, CHOP, neutrophil:lymphocyte ratio, lymphocyte:monocyte ratio, doxorubicin, complete remission, partial remission.

Abbreviations:

CL = Canine lymphoma

NHL = Non-Hodgkin lymphoma

WHO = World Health Organization

DLBCL = Diffuse large B cell lymphoma

mDLBCL-CB = Multicentric, centroblastic diffuse large B cell lymphoma

CR =Complete Remission, PR = Partial Remission, SD = Stable Disease, PD = Progressive Disease.

PFS1= Progression-free survival of the first remission

PFS2 = Progression-free survival of second remission

PFS3 = Progression-free survival of third remission

PFS4 = Progression-free survival of fourth remission

OS= Overall survival

AMC = Absolute monocyte concentration

ALC = Absolute lymphocyte concentration

ANC = Absolute neutrophil concentration

NLR = Neutrophil:Lymphocyte ratio

LMR = Lymphocyte:Monocyte ratio

ROC = Receiver operating characteristic.

AUC = Area under the curve.

Introduction

Canine lymphoma (CL) is one of the most prevalent cancers in dogs and the most prevalent haematopoietic cancer.[1] B-cell immunophenotype and multicentric distribution of disease are the most common presentations.[2–4] CL is considered to be an analogue of the human, Non-Hodgkin's lymphoma (NHL),[5,6] and both the WHO and updated Kiel morphological classifications of NHL have been shown to apply to CL.[7,8] Since the common morphological subtypes of CL involve diffuse effacement of lymph node architecture with a monotypic population of lymphocytes, cytological review of fine needle aspirate samples is considered a sensitive and specific means of diagnosis,[7,9,10] and is the most commonly-used method of diagnosis.[11] Furthermore, cytology has been shown to be a reliable means of applying both the updated Kiel system of morphological classification[8,10,12,13] and the WHO classification.[14–16] The most prevalent cytomorphological subtype of CL (WHO classification) is diffuse large B-cell lymphoma (DLBCL), reported to represent 48% of cases in dogs,[7] and DLBCL can be subdivided in accordance with the Kiel classification into the centroblastic subtype (representing approximately 77% of cases), and the immunoblastic subtype (representing approximately 23% of cases).[7]

Traditionally, outcome for CL has been described in the light of treatment with different chemotherapy protocols. When treated with COP-type protocols, CL cases have been reported to have a median first remission duration of 3-6 months and a 19% one-year survival rate,[17,18] whereas those treated with a CHOP protocol have been reported to have a first remission duration of 8.4-11 months and a one-year survival rate of 50%.[19–22] Nevertheless, these studies have been based on populations of heterogeneous cytomorphological subtypes of CL, and recent studies have shown that heterogeneity in these populations may be a significant confounding factor where prognosis is concerned. Ponce et.al. (2004) demonstrated significant differences in survival between small groups of dogs with different cytomorphological subtypes of CL (using the Kiel classification) and Valli et. al. (2013) later

repeated and expanded these observations using the WHO classification of histological samples.[23] Thus, the response of dogs with specific cytomorphological subtypes of CL to one of the chemotherapy protocols consistently used in the literature is difficult to predict.

Aside from treatment protocol and cytomorphological subtype, many consistent prognostic factors for CL have been reported, for example WHO clinical substage,[19,24,25] immunophenotype,[26–30] histopathological grade,[24,29,31] anatomical location of disease[32–35] and presence of anaemia at diagnosis.[29,36] Other factors such as WHO stage of disease have shown prognostic significance in some studies,[17,37] but not others.[38] Many other features associated with a dog's presentation may also be of prognostic value, but have been less well-evaluated. In human medicine, the pre-treatment absolute monocyte concentration (AMC),[39] absolute lymphocyte concentration (ALC)[40] and the neutrophil:lymphocyte ratio (NLR)[41] have been found to be highly predictive of prognosis in diffuse large B cell lymphoma. Other parameters for example the pre-treatment absolute neutrophil concentration and lymphocyte:monocyte ratio (LMR) have found prognostic significance in other solid tumours.[42,43] In veterinary medicine, AMC[44] and LMR[45] have been found to be of prognostic value in lymphoma, whereas the NLR has not been found to affect prognosis.[46] Pre-treatment absolute neutrophil concentration (ANC) has been found to be prognostic in acute leukaemia,[47]and both AMC and ALC have been found to be prognostic in canine osteosarcoma.[48] The diagnostic utility of NLR in differentiating soft tissue sarcomas from benign neoplasms,[49] and identifying bone marrow involvement in canine mast cell disease has been suggested.[50,51]

The aim of this study was to describe the clinical presentation, response to treatment, progression-free survival (PFS) and overall survival (OS) of a homogenous group of dogs with multicentric, centroblastic diffuse large B-cell lymphoma (mDLBCL-CB) treated with standardised induction and rescue chemotherapy protocols, and to identify prognostic factors associated with this cytomorphological type of CL. Since heterogeneous groups of CL

contain cases with established negative prognostic factors (for example T cell immunophenotype, cutaneous and hepatosplenic presentations), the hypothesis was that a homogenous group of mDLBCL-CB cases will have a longer PFS and OS than a heterogeneous population of CL cases.

Materials & Methods:

Study population: Clinical records of the Royal Veterinary College (RVC) between 2008 and 2016, and North Downs Specialist Referrals (NDSR) between 2010 and 2013, were reviewed retrospectively for multicentric CL cases. Cases were included if they fulfilled the following criteria: a cytological or histological diagnosis of lymphoma from a peripheral lymph node,[4] confirmed B-cell immunophenotype (on flow cytometry, immunohistochemistry or PARR testing), a categorical diagnosis of DLBCL-CB on histopathology or review of cytological findings by a Board-certified clinical pathologist, induction chemotherapy treatment with a COP-type or CHOP protocol, naïve to cytotoxic treatment at the time of mDLBCL-CB diagnosis and where follow-up data to the end of the first remission were available. Cases where therapy was commenced with l-asparaginase or glucocorticoid therapy within 10 days of initiation of cytotoxic treatment were included. Cases of lymphoma that primarily involved extranodal tissue (or the categorization was equivocal), or in which treatment or survival were compromised by a comorbidity (for example end-stage renal failure or advanced congestive heart failure), were excluded. Follow-up information was collected from clinical records of the institutions concerned or obtained from the records of the referring practices.

Data collected: For each case the following data was recorded: age; breed; sex; neuter status; bodyweight; clinical examination findings and concurrent disease status at diagnosis; cytology or histology; immunophenotype; haematology; biochemistry and urinalysis results, and diagnostic imaging findings. Haematology reports were interrogated and the following white blood cell ratios were calculated: neutrophil:monocyte ratio (NMR); neutrophil:lymphocyte ratio (NLR) and lymphocyte:monocyte ratio (LMR). Cases that had concurrent disease which could potentially confound the interpretation of elevated leukocytes (for example evidence of non-cancer-related inflammation detected on physical examination) were excluded from the analysis of the leukocyte concentrations and ratios. Induction

protocols and their associated responses, dates of relapse, details of all rescue protocols used with their associated responses and time of subsequent relapse and the date and reason for death or euthanasia were also recorded.

All available cytological specimens were reviewed by a Board-certified clinical pathologist (BS), to assure that the cytological features were consistent with the diagnosis of DLBCL-CB according to the WHO classification scheme.[14,16] Histopathological classification was also performed where possible by Board-certified anatomical pathologists at the reference laboratories concerned, using the WHO classification. Cases considered not to be DLBCL-CB upon review were excluded. The methods used for immunophenotyping were recorded and immunophenotypic results obtained with flow cytometry were classified as normal or aberrant after Gelain et. al.[52]

Haematology, serum biochemistry and urinalysis results at diagnosis were classified as normal or abnormal according to the reference intervals of the diagnostic laboratories concerned. Haematology results from the chemotherapy treatments during the induction protocols were scrutinized and the incidence and severity of neutropenic episodes (as defined by VCOG CTCAE 1.1)[53] were recorded; a composite "neutropenia score" was then formed by assigning points to each neutropenic event equivalent to the VCOG grade of neutropenia and adding up all points acquired throughout the induction protocol.

Diagnostic imaging findings (computed tomography, radiography, ultrasonography or a combination thereof) at diagnosis were recorded. Using this information the assigned stage and substage according to WHO criteria[4] were reviewed. Bone marrow aspirate or biopsy was not routinely performed and so cases were categorised as presumptive stage V by identification of circulating neoplastic cells in peripheral blood, on review of the blood smear by a board-certified clinical pathologist.

Details of induction and rescue chemotherapy protocols were recorded. Induction protocols were classified as or CHOP (cyclophosphamide, vincristine, prednisolone and doxorubicin), **Supplementary Table 1**, or COP-type (an 8 week induction protocol involving cyclophosphamide, vincristine and prednisolone with or without one subcutaneous dose of cytarabine on the first day of treatment[18]), **Supplementary Table 2**. The CHOP category was comprised of both 19- and 25-week Madison-Wisconsin protocols. Cases needed a minimum of 28 days on a treatment protocol to be classed as either COP-type or CHOP; those who died, were euthanased, or whose treatment was changed less than 28 days after starting a treatment protocol, were classified as having progressive disease and excluded from statistical comparison of treatment protocols. Dogs who achieved remission with COP-type protocols were subsequently treated with the LMP oral maintenance protocol (involving chlorambucil, methotrexate and prednisolone) until disease relapsed; dogs who received the CHOP protocols did not receive maintenance chemotherapy.

Rescue protocols were categorised as DMAC (involving dexamethasone, melphalan, actinomycin-D and cytarabine), **Supplementary Table 3**, COP, doxorubicin-based or lomustine-based, **Supplementary Table 4**. Categories describing a dog's total combination of chemotherapy protocols were defined as: COP-type with no rescue, CHOP with no rescue, COP-type with doxorubicin-based rescue, CHOP with doxorubicin-based rescue, COP-type with non-doxorubicin-based rescue and CHOP with non-doxorubicin-based rescue. The total number of protocols a dog received, and the number of doxorubicin doses each dog received through the entirety of its treatment were recorded. Dogs that received 4 doses of doxorubicin throughout induction and rescue treatment (equivalent to one CHOP protocol) were compared with those that received 1-3 doses (less than a CHOP protocol) and those who received 5-8 doses (equivalent with induction and rescue with CHOP-based therapy). Chlorambucil was substituted for cyclophosphamide in dogs who developed sterile haemorrhagic cystitis. Dogs received echocardiography to monitor their systolic function at

217 the start of doxorubicin therapy; this was repeated at the sixth dose of doxorubicin if the
218 planned cumulative total dose of doxorubicin exceeded 6 doses (180mg/m2).
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220 Response to induction and rescue treatments were evaluated at each visit using VCOG
221 criteria[54] based on physical examination and additional tests at clinician discretion. Cases
222 were classed as complete remission (CR) if lymph nodes had returned to normal size; partial
223 remission (PR) when lymph nodes remained enlarged but had reduced in size by at least 50%
224 and no new lesions were recognized; progressive disease (PD) was used for occurrence of
225 new lesions or increase in size of enlarged lymph nodes by at least 25%; and stable disease
226 (SD) as a change in size of lymph nodes which was not sufficient to be classified as PD or PR
227 with no occurrence of new lesions. The objective response rate was defined as the sum of
228 complete and partial remission rates. A response to treatment must have been maintained for
229 at least 28 days from the date it was first recorded to be classified as CR or PR. Progression-
230 free survival (PFS) was defined as the time from the administration of the first lymphoma
231 treatment (either cytotoxic drug, glucocorticoids or l-asparaginase), to disease relapse,
232 progression or death from any cause; PFS1 was used to denote the PFS for the first remission,
233 PFS2 for the second, and so forth. Dogs that had treatment changed for reasons other than
234 disease progression (for example due to patient's demeanour) were censored from PFS
235 analysis at the point the treatment was altered. Overall survival (OS) was defined as the time
236 between the administration of the first lymphoma treatment and death or euthanasia.[54] Dogs
237 that remained alive at the end of the follow-up period or who were lost to follow-up were
238 censored from OS analysis.
239
240 Statistical analysis: Data were described and analysed using IBM SPSS Version 22 and
241 Graphpad Prism™ (version 6). Data were assessed for normality by visual plotting and use of
242 the Shapiro-Wilk test. Univariable analysis of response to treatment was performed using
243 binary logistic regression; Log-rank and Cox proportional hazard regression were used to
244 analyse the effect of multiple variables on PFS and OS. Where analyses of different

245 treatment protocols were performed, the CHOP or doxorubicin-based groups were used as the
246 comparator, given the acceptance of doxorubicin-based protocols as the standard of care for
247 CL therapy.[20] The alpha level was set at P=0.05 for significance. ROC curve analysis was
248 performed to assign cut-off values to continuous variables used for the prediction of a PFS or
249 OS exceeding a certain time, usually its median value; a minimum area under the curve of 0.7
250 was required for the ROC model to be considered. Comparison of groups with different
251 survival times was performed using Fisher's exact test for categorical variables. Student's
252 (two-tailed) t-test and Mann-Whitney U test for parametric and non-parametric continuous
253 variables respectively.
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Results

Clinical Presentation

Search results revealed 91 cases of canine B cell lymphoma; out of these, 65 cases of multicentric CL were identified and 45 dogs were classified as mDLBCL-CB by the same board-certified clinical pathologist; 35 dogs from the RVC and 10 from NDSR. Thirty-one breeds were represented, and the most common were cross-breeds (8 dogs), Jack Russell terriers (4), golden retrievers (2), beagles (2), West Highland white terriers (2) and flat-coated retrievers (2). The mean age was 8.1 years (standard deviation 2.63 years) and the median bodyweight was 28kg (range: 3.4-59.5kg). Seventeen dogs (38%) were female (16 neutered, 1 entire), and 28 (62%) were male (18 neutered, 10 entire). Median follow-up period was 316 days (range 6-1375 days); at the end of the study period, 36 dogs had died or been euthanized due to lymphoma, 4 had been euthanased for another reason, 4 dogs remained alive and 1 dog was lost to follow-up. Reasons for euthanasia other than lymphoma were cellulitis (1 dog) and heart failure (3 dogs). The dog that developed cellulitis was euthanased 6 days after starting treatment and the dogs that developed heart failure were euthanased 316 days, 790 days and 1297 days after starting treatment.

The key characteristics of the populations of dogs from the Royal Veterinary College and North Downs Specialist Referrals were not significantly different. All dogs resided in the South-East of England, and most lived in urban areas. All dogs were owned by fee-paying clients and none were referred to partake in clinical trials. Both groups of dogs had approximately equal gender representation (RVC 70% male versus NDSR 60% male), bodyweight (RVC median bodyweight 28kg versus NDSR 27kg) and age (RVC median age 8yo versus NDSR 9yo). There was a similar distribution of disease stage, but a greater proportion of RVC dogs presented as substage a (62%, versus 40% for NDSR). None of these differences were statistically significant ($P>0.05$).

Haematology findings from before the administration of anticancer treatment (including steroids or l-asparaginase) were available for 29 dogs. Eleven dogs (38%) were anaemic (median PCV 33%, range 29-37%, all non-regenerative or insufficiently-regenerative), 7 dogs (24%) had a neutrophilia, 12 dogs (41%) were lymphopenic, one dog (3%) had monocytosis and one dog (3%) had a lymphocytosis. Median absolute leukocyte concentrations and ratios are displayed in **Table 1**. Pre-treatment serum biochemistry and urinalysis were available for 35 dogs; 10 (29%) had biochemical abnormalities, the most common of which were hyperbilirubinaemia and increased activities of serum ALT and ALP in 4 dogs (11%) and elevated cholesterol in 3 dogs (9%). All dogs with hyperbilirubinaemia were stage 4 or 5. Five of 35 dogs had abnormalities detected on urine dipstick; bilirubinuria was observed in the 4 dogs with hyperbilirubinaemia (11%) and proteinuria (defined as at least 2+ protein on dipstick analysis, in association with an unreactive urinary sediment, a urine specific gravity of less than 1.030, and the absence of haematuria) was seen in 3 dogs (9%).

Immunophenotyping was performed by flow cytometry in 37 dogs (82%), by immunohistochemistry in 7 dogs (16%), and by PARR in 1 dog (2%). Full flow cytometry results were available for 32 dogs; for 5 dogs, only the immunophenotype was available, details of the markers run and graphs were unavailable for scrutiny. A comprehensive set of markers (including CD3, CD5, CD21, CD34, CD45 and CD79a) was available for 23 dogs. Out of these, 12 dogs (52%) had a normal immunophenotype (consisting of positive labelling with CD21, CD79a and CD45) and 11 dogs (48%) had an aberrant immunophenotype; two dogs (18%) were classified as aberrant phenotypes due to the additional co-expression of T cell markers (CD3 in one dog and CD5 in the other), whereas the remaining 9 dogs (82%) had additional co-expression of CD34 or absent expression of CD21, CD79a, or CD45. Two dogs had more than one aberration (**Table 2)**. The two dogs that expressed CD34 were classed as lymphoma cases rather than acute leukaemias because of the absence of both cytopaenias and circulating neoplastic cells.

Stage was documented for 35 dogs (78%). Twenty-one dogs (60%) had thoracic and abdominal imaging, 13 with CT and 8 with thoracic radiographs and abdominal ultrasonography; 6 dogs (17%) had abdominal ultrasonography without thoracic imaging. Nineteen dogs (54%) had fine needle aspiration cytology of the liver and spleen. Eight dogs (23%) were categorised as stage 3, 18 (51%) as stage 4, and 9 (26%) as stage 5; (four with circulating neoplastic cells and 5 with extranodal tissue involvement). Substage information was available for 44 dogs; 25 (57%) were substage a and 19 (43%) were substage b.

Induction Treatment and Response

Twenty-seven dogs (60%) were treated with CHOP induction, versus 15 (33%) COP-type; 3 dogs (7%) had progressive disease or death below 28 days, making them ineligible for placement in COP-type or CHOP treatment groups. Nine dogs (20%) were pre-treated with l-asparaginase, 3 before a COP-type protocol and 6 before a CHOP protocol (median time before the protocol started was 1 day). Three dogs (7%) were pre-treated with prednisolone (median time before protocol started 3 days). Haematology nadirs after chemotherapy treatments in the induction protocols were available for 25 dogs; of these, 14 dogs (56%) developed at least one episode of neutropenia, and the median neutropenia score for these dogs was 3 (range 1-13). A greater proportion of dogs from NDSR were induced with a CHOP protocol (80%) than RVC (60%), however this difference was not statistically significant (P=0.286).

The objective response rate to induction treatment was 94%; 34 dogs (76%) obtained a CR, 8 dogs (18%) obtained a PR, and three dogs (6%) developed PD. Ninety percent of entire dogs had complete responses to treatment whereas 55% of neutered dogs had complete responses to treatment (P=0.017); no other variables were found to have a significant effect on response to induction therapy. Thirty-seven dogs (82%) completed the induction protocol, 7 dogs (16%) had progressive disease during induction treatment, and one dog's (2%) induction

protocol was changed while it had a partial response to treatment due to the dog's aggressive behaviour.

First Remission Progression-Free Survival (PFS1)

Median PFS for the first remission (PFS1) was 182 days (95% CI: 144-228 days). There was no difference in PFS1 between the two institutions (P=0.710). Dogs that were not anaemic at presentation had a significantly longer median PFS1 (254 days, 95% CI: 189-319 days) than those that were anaemic on presentation (median 147 days, 95% CI: 22-272 days; P=0.002, **Fig.1)**. Dogs with a neutrophil:lymphocyte ratio below a cut-off value of 9.44 had longer median PFS1 (216 days, 95% CI: 138-294 days) than those with NLR above 9.44 (median PFS1 104 days, 95% CI: 0-213 days; P=0.015). The cut-off value of 9.44 had a 84% sensitivity and 64% specificity of predicting the probability of PFS1 exceeding its median value, 182 days (AUC 0.75). Dogs with a neutropenia score above 1.5 during induction therapy had a significantly longer median PFS1 (254 days, 95% CI: 141-367 days), than dogs with a score below 1.5 that had a median PFS1 (216 days, 95% CI: 38-394 days; P=0.049). The cut-off value of 1.5 had a sensitivity of 50% and specificity of 86% for predicting the probability of PFS1 exceeding its median of 182 days (area under the curve 0.7). Use of a COP-type protocol provided shorter median PFS1 (147 days, 95%CI: 73-221 days) than CHOP (median PFS1 251 days, 95% CI: 215-293 days; P=0.000, **Fig.2**) and achieving a complete response was associated with longer PFS1 (246 days, 95% CI: 183-309 days) than a partial response (median 105 days, range 32-178 days; P=0.003). No other variables were found to have any significant effect on PFS1.

On multivariable analysis, only the absence of anaemia at diagnosis (P=0.005) and treatment with a CHOP protocol over a COP-type protocol (P=0.008) were found to be independently predictive of a longer PFS1.

Rescue Therapy and Subsequent Progression-Free Survival

Out of the 42 dogs (93%) that responded to induction treatment, 39 dogs (93%) relapsed, two dogs (5%) remained in remission at the time of study completion, and one dog (2%) was lost to follow-up. The three dogs (7%) that had progressive disease with induction therapy did not receive rescue therapy, and were euthanased. For the 37 dogs (82%) that completed the induction protocol, the median time between protocol completion and disease relapse was 118 days (range 7-309 days); 120 days (range 7-309 days) for dogs treated with CHOP protocol, and 49.5 days (range 7-208) with COP-type (P=0.01). Twenty-five dogs (55%) received rescue protocols, comprised of 14 (56%) doxorubicin-based, 6 (24%) COP-based protocols, 3 (12%) DMAC and 2 (8%) lomustine-based. Ten dogs (67%) that received COP-type induction had rescue treatments, versus 15 (55%) that received CHOP; the distribution of rescue treatments is illustrated in **Table 3**. A greater proportion of dogs from RVC received rescue protocols (73% versus 40% from NDSR, P=0.024).

The objective response rate to the first rescue protocol was 86%; 15 dogs (68%) achieved CR, 4 dogs (18%) achieved PR, 3 dogs (14%) developed PD. The median PFS2 was 147 days (95% CI: 104-190 days). The only variable with a significant effect on PFS2 length was response to treatment; dogs who had a CR had a median PFS2 of 166 days (95% CI: 132-199 days), dogs that had a PR had median PFS2 56 days (95% CI: 25-87 days) and those who didn't respond to treatment had a median PFS2 of 14 days (range 1-14 days; P=0.000).

Fifteen dogs received a second rescue protocol. Protocols used were: 6 (40%) lomustine-based, 4 (27%) doxorubicin-based, 4 (27%) DMAC, and 1 (6%) COP. The objective response rate was 28%; 2/14 dogs (14%) each obtained a CR, PR and SD, whereas 8 dogs (58%) developed PD. Median PFS3 was 23 days (95% CI: 9-37 days). Eleven dogs received a third rescue protocol; 5 (45.5%) were lomustine-based, 3 (27.5%) were DMAC, 2 (18%) received a clinical trial drug and 1 (9%) was doxorubicin-based. The objective response was 60%; 2/10 dogs (20%) obtained CR, 4 dogs (40%) a PR, while 2 dogs each maintained SD and developed PD. Median PFS4 was 32 days (95% CI: 18-46 days). Four dogs received a

fourth rescue protocol; one case received a clinical trial drug, one received DMAC, one masitinib and the other received alternating doses of bleomycin and mitoxantrone. All maintained SD and the median PFS5 was 39 days (range 28-111 days). Statistical analysis of factors influencing the duration of PFS3, PFS4 and PFS5 was not performed due to small group sizes.

Overall, 5 dogs (12%) received a COP-type induction protocol with no rescue, and 12 dogs (29%) received a CHOP induction protocol with no rescue. Eight dogs (19%) received a COP-type induction followed by rescue protocols which contained doxorubicin, 2 dogs (5%) received COP-type induction followed by rescue protocols which did not contain doxorubicin, 9 dogs (21%) received CHOP induction followed by rescue protocols which contained doxorubicin, and 6 dogs (14%) received a CHOP induction followed by rescue protocols which did not contain doxorubicin.

The median number of treatment protocols received was 2 (1-5). Thirty-seven dogs (82%) received doxorubicin at some point during their overall treatment. The median number of doxorubicin doses for those dogs receiving this drug at some point in induction and rescue therapy was 4 (range 1-8; **Table 4**); the total number of doxorubicin doses did not differ significantly between dogs from RVC and NDSR (P=0.259). The 3 dogs who were euthanased due to heart failure had a total number of 3, 4 and 5 doses of doxorubicin. No cardiotoxicity was seen in the 8 dogs that received more than 5 doses of doxorubicin (total dose exceeding 150mg/m2).

<u>Overall Survival (OS)</u>

The median OS for all dogs was 322 days (95% CI: 259-385 days). No significant difference in OS was found between the two institutions (P=0.366). OS was significantly shorter for large dogs (P=0.010); using an ROC (AUC 0.73) a cut-off value of 31.15kg was found, with 82% sensitivity and 70% specificity for predicting OS of less than the median of 322 days.

Dogs with a lower NLR (P=0.047) and a higher LMR (P=0.020) had a longer OS; using ROC analysis a LMR with a cut-off of 1.43 was found with a sensitivity of 83% and specificity of 63% for predicting OS greater than the median (AUC 0.72), **Fig. 3**, and a cut-off for NLR of 11.44 predicted survival less than 90 days with 80% sensitivity and 100% specificity (AUC 0.87), **Fig. 4**.

Dogs that attained CR with induction therapy had a significantly longer OS (median 400 days, 95% CI: 291-509 days) than dogs that attained a PR (median OS 169 days, 95% CI: 57-281 days; P=0.037). Dogs that attained CR with the first rescue protocol had a significantly longer OS (median OS 493 days, 95% CI: 383-603 days) than those that developed PD or SD (median OS 216 days, 95% CI: 86-346 days; P=0.009), but there was no significant difference in OS between dogs that had a CR or a PR (median OS 422 days, range 214-630 days; P=0.693). Dogs that received CHOP induction had a median OS of 401 days (95% 258-544 days) whereas dogs that had a COP-type induction had a median OS of 257 days (95% CI: 157-357 days); this difference was not significant (P=0.313)

Dogs that received rescue protocols had a significantly longer OS (median 401 days, 95% CI: 288-514 days) than dogs that received no further treatment when lymphoma recurred (median OS 227 days, 95% CI: 132-322 days; P=0.009). However, the total number of treatment protocols a dog received had no significant effect on OS (P=0.351). Dogs that received COP-type induction and no rescue therapy had a median OS of 192 days (95% CI: 153-231 days) compared with 316 days (95% CI: 219-413 days) for dogs that received a CHOP protocol and no rescue (P=0.024). No significant difference in OS was found between dogs that received a CHOP induction protocol with no rescue therapy, and those that received COP-type induction followed by doxorubicin-based rescue therapy (P=0.213). No significant difference in OS was found between dogs that received a CHOP induction protocol with no rescue therapy and dogs that received a CHOP induction followed by non-doxorubicin-based rescue therapy (P=0.925); however, dogs that received CHOP induction followed by

doxorubicin-based rescue therapy had a significantly longer OS (median 706 days, 95% CI: 350-1062 days) compared with CHOP induction alone (P=0.04; **Fig. 5**).

Overall, the number of doxorubicin doses (from 0-8) was highly significantly associated with longer overall survival (P=0.000). When dogs that were not given doxorubicin were excluded from analysis, the strong association remained (P=0.002, **Table 5**). Dogs that received 1-3 doses of doxorubicin had a median OS of 216 days (95% CI: 24-408 days), which was not significantly different to dogs that had 4 doses, equivalent to a complete CHOP protocol (median OS 322 days, 95% CI: 280-364 days, P=0.777), but significantly shorter than dogs that had 5-8 doses (median OS 706 days, 95% CI: 297-1115 days, P=0.049, **Fig. 6**).

The two dogs with expression of the T cell markers CD3 and CD5 had a subjectively shorter OS of 32 and 262 days compared with the rest of the study group (median OS 422 days, 95% CI: 260-584 days; P=0.031). No such apparent difference in OS was noted with other aberrations in immunophenotype.

On multivariable analysis, the variables found to be independently predictive of OS were NLR (P=0.009), LMR (P=0.031), and the combination of induction and rescue protocols (P=0.030) and the number of doxorubicin doses (for the dogs who received doxorubicin, P=0.002). No other variables had a significant effect on OS.

Prolonged and Short Survival Groups

In this study, the 6-month survival rate was 73%, the 1-year survival 38% and the 2-year survival rate was 9%. Dogs living more than 1 year had lower median bodyweight (mean 22.5kg versus 31kg, P=0.019), were less likely to be anaemic on presentation (prevalence of anaemia at diagnosis of 11% versus 53%, P=0.049), had a CR with induction therapy (100% versus 64%, P=0.007) and when the dogs who received doxorubicin were considered, those

who received more doxorubicin doses were more likely to survive greater than one year (median number of 5 doxorubicin in dogs living greater than one year compared with 4 doses, for those that lived less than 1 year, P=0.031). Statistical analysis of dogs living longer than 2 years was not performed due to small group size.

Dogs with shorter survivals than 6 months had lower rates of CR with induction therapy (42% versus 91%, P=0.001), were older (mean age of 9.5 years versus 7.6 years, P=0.031) and less likely to be treated with rescue therapy (27% receiving rescue versus 69%, P=0.031) than those who lived over 6 months.

Discussion

In this study, a homogenous population of mDLBCL-CB cases are described. The median PFS1 and OS of the whole group (182 days and 322 days respectively), and proportions surviving to 1 and 2 years (38% and 9% respectively) were lower than[19,55,56] or similar to[57,58] previous reports of groups containing mixed subtypes of multicentric CL. The OS of dogs in this study may not have been greater than that reported in mixed groups because many previous studies have been designed to evaluate a specific chemotherapy protocol, whereas this study evaluated a specific subtype of disease. A homogeneous population also lacks the potential influence of subtypes of disease that have much longer survival times for example T-zone lymphoma,[59] or shorter survival times for example lymphoblastic lymphomas.[23] The level of treatment a dog received varied in this study, including dogs that were given treatments which are not considered the "standard of care," prescribed for reasons such as finance or owner convenience. When specific combinations of induction and rescue therapy are evaluated, greater OS times are seen, for example dogs who received CHOP induction followed by doxorubicin-based rescue had a median OS of 706 days (95% CI: 350-1062 days).

The response to induction treatment of mDLBCL-CB (objective response rate of 94%, complete response rate of 76%) was high. This rate has been similar in some studies[18,58,60] and lower in others.[19,55,56] Since mDLBCL-CB is the most prevalent type of lymphoma in dogs,[7] a level of similarity between this study group and a random selection of CL cases would be expected. Studies of mixed immunophenotype groups are likely to have included dogs with less responsive variants of the disease for example indolent lymphomas, that may not achieve complete responses.[59] In this study group, entire dogs had a significantly higher rate of complete responses to induction therapy, however a greater proportion of entire dogs were treated with CHOP induction therapy than neutered dogs (73% versus 61%) and the low number of entire dogs (n=11) throws the independence of this finding into question.

The presence of anaemia at diagnosis was shown to be associated with a significantly shorter PFS1 and was significantly more common in the group of dogs who survived less than one year, compared with dogs that survived over 1 year. The negative prognostic value of anaemia in canine lymphoma has been demonstrated in previous studies,[36,61,62] although the aetiology is unclear. Bone marrow infiltration by neoplastic lymphocytes has been hypothesized. However, one study found no significant difference in occurrence of marrow infiltration between groups of anaemic and non-anaemic dogs with lymphoma, and no significant difference in haematocrit between dogs with marrow involvement and those without.[62] Anaemia of inflammatory disease is another potential aetiology. A previous study has shown no laboratory evidence of this in a group of dogs with lymphoma, although decreased response of the bone marrow to erythropoietin is still possible.[63] The pathophysiological mechanism associating anaemia with a poor prognosis is unclear. One theory suggests that the state of chronic hypoxia may induce expression of proteins which enable cancer cells to deal better with stress; increased concentration of one such protein, vascular endothelial growth factor has been associated with a poorer prognosis in canine lymphoma,[64] and both anaemia and a poorer prognosis in humans with NHL.[65]

Choice of a CHOP induction protocol over a COP-type protocol was shown as independently prognostic for PFS1 in this study, in line with previous publications.[18] The superior PFS1 associated with the CHOP protocol may be related to both the longer duration of treatment and the use of another agent, doxorubicin. In agreement with other publications,[66,67] this study found an association between the development of neutropenia and PFS1. Since data on the development of neutropenia were only available for fewer than half of the dogs, and the prevalence of neutropenia among these dogs was low, we believe that assessment of the prognostic effect of neutropenia in this study was statistically under-powered. The prevalence of neutropenia may have been low because dogs induced with a COP-type protocol would

have received a less immunosuppressive and shorter induction period compared to other studies, and because dose reductions were often performed following neutropenic episodes.

Response to the first rescue protocol (86%) and the median PFS2 (147 days) were similar to the response to induction therapy (94%) and the median PFS1 (182 days). Such findings have been described before.[68] For a number of dogs in this study group, the first rescue protocol can be regarded as "re-induction" since the median time between completing induction therapy and relapse was 118 days, making the development of appreciable drug resistance less likely, and secondly a number of dogs who were induced with a COP-type protocol received a doxorubicin-based rescue therapy. The response rates and remission durations associated with the second, third and fourth rescue protocols were more similar to previous reports of rescue treatment for lymphoma when drug-resistance is established.[69–72]

This study has shown the independent prognostic significance of the pre-treatment neutrophil:lymphocyte ratio (NLR) in predicting progression-free survival and OS in canine mDLBCL-CB. Absolute leukocyte counts and their ratios are ways of measuring different aspects of systemic inflammation and in cases of cancer systemic inflammation may be caused by tumour-related inflammation.[43] Tumour-related inflammation is associated with cancer progression[73] and in human medicine NLR has been recognised as holding prognostic significance in diffuse large B cell lymphoma,[41] and many solid tumours.[43] In veterinary medicine, NLR has been shown to be of some prognostic significance (on univariable but not multivariable analysis) in canine mast cell tumours.[74] Mutz et.al recently reported no prognostic significance of NLR in a study of canine multicentric lymphoma treated with a CHOP protocol.[46] If the findings of our study are supported by subsequent work, the apparent disagreement with the study of Mutz et.al may be due to an innate feature of mDLBCL-CB and the homogenous population of mDLBCL-CB cases in this study. Conversely, increased NLR is a very non-specific finding and despite screening dogs for concurrent disease, it is

possible that the results are confounded by dogs with sub-clinical benign conditions, for example dental disease, otitis externa, or pancreatitis.

We have reported that high NLR values (above a cut-off of 9.44 for PFS1 and 11.44 for OS), correspond to a poor prognosis. In human medicine, a cut-off value of 3.5 is established, above which DLBCL patients have both a significantly poorer PFS and overall survival.[41] Such high NLR cut-off values may make this test insensitive in veterinary medicine; however it may also reflect that neutrophilia and lymphopenia due to stress is more common in canine patients than humans, and so significant NLR levels will need to be in excess of that expected from a normal stress leukogram. Previous veterinary studies have evaluated absolute monocyte and lymphocyte counts in cancer; prognostic significance has been shown in canine osteosarcoma,[48] and in some studies of canine lymphoma[44] but not others.[46] LMR has previously been reported as prognostic in human NHL,[75] in canine mast cell disease[74] and in canine lymphoma.[76] This study concurs with the previous report of the prognostic significance of LMR for survival in mDLBCL-CB. However just as for the significance of NLR, caution is needed in interpreting LMR values due to the non-specific aetiologies of its elevation.

This study has demonstrated that use of a rescue protocol, particularly rescue therapy involving doxorubicin, and the total number of doxorubicin doses received to be a strong prognostic factor in mDLBCL-CB cases. These observations should be interpreted with caution as many dogs who received a higher number of doses of doxorubicin are likely to be owned by the most committed owners, and subsequent doxorubicin treatments were unlikely to be offered unless the dog had responded to the drug previously. Thus, dogs who received the most doxorubicin may have been selected by lack of drug resistance relative to the other dogs. This could highlight an innate tractability in a subset of dogs with mDLBCL-CB which has yet to be identified. The lack of significantly different OS between dogs that received a COP-type induction followed by doxorubicin-based rescue therapy and dogs that received a

CHOP induction protocol (with or without non-doxorubicin-based rescue therapy) suggest that the temporal placement of doxorubicin in either a dog's induction or rescue treatment does not seem to be of prognostic value. Being able to give a COP-type protocol for induction therapy without the concern of undermining prognosis may offer flexibility in choice of induction protocols to veterinarians and their clients.

Aberrant immunophenotypes of CL have been described based on flow cytometry,[52,77] although their prognostic significance is largely uncertain. Possession of an aberrant immunophenotype overall held no prognostic value in this study, however statistical analysis of the particular aberrations present was not possible due to small group sizes. The poorer OS experienced by the two cases that expressed T cell markers is interesting; further work is necessary to investigate different prognoses conferred by different aberrations of immunophenotype.

In this study group, the majority of dogs presented in clinical stage 4 or 5, and almost half of them in substage b. These proportions may be normal for mDLBCL-CB, or the apparent bias towards more advanced disease may be a result of thorough application of staging tests and the phenomenon of stage migration[38] or selection bias since all cases were recruited at specialist referral centres. Stage, substage, gender, and bodyweight have been previously reported as prognostic in canine lymphoma[17,19,36,37,61,67] and a previous publication has reported a different behaviour of stage 5 cases with extranodal involvement compared with those with haematological involvement.[55] None of these findings were supported by this study. Prognostic significance of clinical stage in previous, heterogeneous groups of lymphoma cases may have been confounded by the inherent variability of different CL subtypes. Equally, the prevalence of certain factors was too low in the current study for meaningful inclusion in statistical analysis.

Different factors were found to have prognostic significance for PFS1 and OS. Although the application of some variables to PFS1 would be meaningless, the discrepancy between the prognostic significance of LMR, response to induction therapy and the presence of anaemia at diagnosis for PFS1 versus OS is harder to explain. These findings may have arisen due to the population size and the effect of censoring some cases from OS analysis which were included in PFS1 analysis. Nevertheless if these findings hold true in larger populations one explanation could be that features such as some absolute cell concentration or ratios reflect the susceptibility of the disease to treatment at that point in time, thus they may be prognostic for length of the subsequent remission only, rather than for OS.

In this study, the authors feel justified in combining two different hospital populations since they are not significantly different in any key feature of clinical presentation or induction therapy, and their cytology has been reviewed by the same board-certified clinical pathologist. The only significant difference between the two groups is that a higher proportion of cases from RVC received a rescue protocol; we feel that this doesn't make the combination of populations any less valid since the choice of whether to use rescue therapy or not is dependent on the owners' wishes and finances, and it is not possible for these variables to be well controlled in retrospective studies. The finding of this study with regard to rescue therapy was that a response to rescue therapy is positively prognostic, and this is corroborated by other studies.[68]

This study was limited by its retrospective nature and relatively small population size; resultantly the study is most likely underpowered and significant findings may have been missed. A large number of variables have also been statistically evaluated, giving the possibility that false positive associations have arisen by chance. Larger multi-institution studies are clearly needed to clarify this study's findings. The recruitment of cases from speciality referral centres might have also biased the caseload to those which had more complicated presentations, more likely to be of substage b, and with more committed owners.

Although the study describes the response to different treatments, the authors caution that a retrospective study represents weak evidence to guide future therapeutic decisions. We hope that this study provides grounds for prospective controlled trials in this area. Investigation of the genetic differences within this group of dogs may help to predict the dogs that will develop drug resistance sooner versus those will demonstrate prolonged response to therapy.[78]

In conclusion, this study has shown the behaviour of disease and response to certain drugs in a population of dogs with mDLBCL-CB. Absence of anaemia at diagnosis and a pre-treatment NLR below 9.44 were associated with longer PFS1 while LMR above 1.43, and NLR below 11.44 were associated with longer OS. The use of rescue therapy and the number of doxorubicin doses received were strongly associated with longer OS. The choice of induction protocol did not influence survival, providing doxorubicin was later used as rescue therapy. Further, prospective studies are warranted to further assess the importance of these findings.

Acknowledgements :

I would like to thank Dr. Ruby Chang (Royal Veterinary College, UK) for advice on statistical analysis.

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Legends to Tables & Figures

Table 1: Pre-treatment absolute leukocyte concentrations and ratios from the study population (n=25). *****The two ratios which were found to have a significant effect on outcome have been marked in bold; NLR was independently predictive for PFS1 in (*P*=0.025), and OS (*P*=0.009). LMR was independently predictive of OS (*P*=0.031).

Table 2: Signalment and cellular markers of dogs with aberrant immunophenotypes. The aberrant markers for each dog are placed in bold. "+" = present, "-" = absent. FN = female neutered, ME = male entire, MN= male neutered. Statistical analysis of individual aberrations was not performed due to small sample sizes.

Table 3: Rescue protocols used after COP-type and CHOP induction protocols (n=42).

*****Bleomycin / mitoxantrone.

#Masitinib.

Table 4 : Distribution of the total number of dogs receiving different numbers of doxorubicin doses and the placement of doxorubicin therapy through induction and rescue treatment.

Table 5 : Significant differences between the total number of doxorubicin doses received and OS. Median OS values are given for each dose. Significant p values are marked in bold. Hazard Ratios (HR) are given for categories where significant differences in OS were found.

*Only one dog received 7 doses of doxorubicin hence the OS value is actual rather than median.

Figure 1: Kaplan Meier curves for the first progression free survival times (PFS1) of dogs with mDLBCL-CB (n=28); presence of anaemia at diagnosis was independently predictive of the length of first remission on cox hazard regression. Dogs who were anaemic at diagnosis

(dotted line) had a median PFS1 of 147 days (95% CI: 22-272 days) and an increased hazard of remission ending (hazard ratio 5.6) compared with those that were not anaemic on presentation that had a median PFS of 254 days (95% CI: 189-319 days), *P*=0.002.

Figure 2: Kaplan Meier curves for the first progression free survival times (PFS1) of dogs with mDLBCL-CB (n=42); induction protocol was independently predictive of the length of first remission on cox hazard regression. Dogs who were treated with the CHOP protocol (solid line) had a median PFS1 of 251 days (95% CI: 215-293 days) and decreased hazard of remission ending (hazard ratio 0.22) compared with a median PFS1 of 147 days (95%CI: 73-221 days) for those that were treated with the COP-type protocol (*P*=0.000).

Figure 3: Kaplan Meier curves for the overall survival times (OS) of dogs with mDLBCL-CB (n=28); pre-treatment lymphocyte:monocyte Ratio (LMR) was independently predictive of OS length on cox hazard regression (*P*=0.031). Dogs who had a LMR above a cut-off of 1.43 had a median OS of 353 days (95% CI: 208-498 days), while those who had an LMR below 1.43 had a median OS of 174 days (94-254), *P*=0.01; hazard ratio 0.315.

Figure 4: Kaplan Meier curves for the overall survival times (OS) of dogs with mDLBCL-CB (n=28); pre-treatment Neutrophil : Lymphocyte Ratio (NLR) was independently predictive of OS length on cox hazard regression (*P*=0.009). Dogs who had a NLR above a cut-off of 11.44 had a median OS of 128 days (0-325), while those who had an NLR below 11.44 had a median OS of 322 days (95% CI: 241-403 days); *P*=0.000, hazard ratio 7.7.

Figure 5: Kaplan Meier curves for the overall survival times (OS) of dogs with mDLBCL-CB (n=28); combination of total treatment was independently predictive of OS length on Cox hazard regression (*P*=0.030). In comparison with dogs that received CHOP induction followed by no rescue therapy, dogs who received a COP-type protocol with no rescue had an increased hazard ratio for death of 4.2 (*P*=0.024), whereas dogs who received CHOP

induction followed by doxorubicin-based rescue therapy had a reduced hazard ratio for death of 0.330 (P=0.04). No significant differences were seen between dogs that received COP-type induction followed by doxorubicin-based rescue, dogs that received CHOP induction with no rescue protocol and dogs that received CHOP induction followed by a non-doxorubicin-based rescue protocol. The two dogs that received COP-type rescue after COP-type induction have been admitted from this graph for clarity.

Figure 6: Kaplan Meier curves for the overall survival times (OS) of dogs with mDLBCL-CB (n=37); the number of doses of doxorubicin received throughout induction and all rescue therapy was independently predictive of OS on cox hazard regression (P=0.002). Compared with 1-3 doses of doxorubicin, dogs who received 5-8 doses had a decreased hazard ratio of death of 0.399 (P=0.049). There was no statistically significant difference between dogs that received 4 doses and dogs that received 1-3 doses.

Supplementary Table 1: The CHOP protocol. The Madison Wisconsin 19-week CHOP protocol consisted of 4 repetitions of the above cycle of cytotoxic drugs; a tapering course of prednisolone is given in the first cycle, with discontinuation of the drug at the start by the start of the second cycle. The Madison Wisconsin 25-week CHOP protocol involved a two-week break between cytotoxic drug treatments in the third and fourth cycles. No maintenance chemotherapy was given with this protocol. *mg/kg dosing was used for doxorubicin below a bodyweight of 10kg.

Supplementary Table 2: The COP-type protocol. Prednisolone was given throughout induction and maintenance phases of the protocol. *A single dose of cytarabine was given subcutaneously on the first day of the treatment in some dogs.

Supplementary Table 3: The DMAC Protocol. This protocol consists of ongoing 2-week cycles as described in the table.

994 **Supplementary Table 4:** The single-agent protocols.

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