**Evaluation of a multi-agent chemotherapy protocol combining dexamethasone, melphalan, actinomycin D, and cytarabine (DMAC) for the treatment of resistant canine non-Hodgkin high-grade lymphomas: a single centre's experience**

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**Running Head**

DMAC protocol for resistant canine lymphoma

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**Abstract**

The DMAC protocol (dexamethasone, melphalan, actinomycin-D, cytarabine) has been evaluated in American studies for the treatment of relapsed canine lymphoma, comparing similarly to other rescue protocols. The aim of this study was to evaluate efficacy and toxicity of DMAC, in a larger United Kingdom cohort of resistant canine lymphomas. Medical records of dogs with resistant non-Hodgkin high-grade lymphomas that received DMAC as a rescue protocol were reviewed from 2007-2017. Response, time from initiation to discontinuation (TTD), and toxicity (VCOG criteria) were assessed. One hundred dogs were included; 86 received CEOP (modified CHOP including epirubicin) as first-line treatment. Thirty-five dogs (35%) responded: 21 complete responders (CR) and 14 partial responders (PR). Responders had significantly longer TTD (p<0.001) compared to non-responders: 62 days (range 28-952) for CR versus 32 days (range 20-70) for PR. Six CR received more than six cycles of DMAC (range 7-36 cycles) and experienced a longer TTD (median 508, range 126-952 days). Thrombocytopenia occurred in 45% (24 grade 1-2, 21 grade 3-4) and neutropenia in 36% of cases (29 grade 1-2, 7 grade 3-4). Gastrointestinal toxicity occurred in 42% of dogs (40 grade 1-2, 2 grade 3-4). Due to chemotherapy toxicity, treatment was discontinued in 5, and hospitalisation required in 6 cases. In this study, response to DMAC was lower and of generally shorter duration than previously reported. Toxicity was high, but infrequently led to hospitalisation or discontinuation of treatment.

*Keywords:* Canine lymphoma; resistant lymphoma; DMAC; actinomycin-D; melphalan; dog; chemotherapy; rescue

# **Introduction**

Haematopoietic neoplasia is common in dogs, with non-Hodgkin lymphomas (cNHLs) making up over 80% of all hematopoietic cancer.1,2 Among cNHLs, high-grade B-cell lymphomas prevail and first-line chemotherapy usually consists of maintenance free protocols including prednisolone, vincristine, cyclophosphamide and doxorubicin (CHOP).2-7 A CHOP variant including epirubicin (CEOP) has also been published showing very similar results.8 High-grade T-cell lymphomas are less common and though response rates and duration are shorter, treatment is similar.9 The overall response rate for CHOP/CEOP protocols is between 89-100% and the median ﬁrst remission duration is 142 to 302 days.3-5,7,8 Median ﬁrst remission duration for T-cell cNHL is shorter than 5 months, compared to 11 months for B-cell cNHL.10

In almost all cases, development of drug resistance leads to disease progression and recurrence. Rescue chemotherapy protocols are used following failure to re-induce remission with the first-line protocol, or relapse during first-line therapy. Several single-agent and multi-agent rescue protocols are described; response rates are highly variable (18-77%) and remission durations are shorter in comparison to first-line treatment (56-238 days).11-23 Evaluation of the efficacy and tolerability of rescue protocols is limited by the relatively small number of cases in comparison to studies of first-line protocols and the significant variability in the degree of pre-treatment.

The DMAC protocol is a multi-agent chemotherapy protocol combining dexamethasone, melphalan, actinomycin-D, and cytarabine. Actinomycin-D, cytarabine, and dexamethasone are administered during week 1 of the protocol, followed by melphalan and dexamethasone during week 2.14,16 Alvarez *et al.* reported an overall response rate of 72% with a median duration of remission of 61 days.16 Parsons-Doherty *et al.* reported lower efficacy, with an overall response rate of 43% and median overall progression free survival of 24 days.14 Factors reportedly associated with response to DMAC included previous treatment with doxorubicin, response to previous protocols, and number of previous relapses. The most commonly reported adverse event has been thrombocytopenia (41-56% cases; 39-61% grade 3-4) and less commonly neutropenia (17% cases; 47-100% grade 3-4) and gastrointestinal toxicity (13-22% cases; 89-90% grade 1-2). 14,16

The discrepancy in efficacy reported by the current studies highlights the need for further evaluation of the DMAC protocol. The aim of this study was to evaluate the efficacy and tolerability of the DMAC protocol in resistant high-grade cNHL treated at a single referral centre. A second aim was to identify predictive factors for response and toxicity.

# **Materials and methods**

## *Study population*

The computerised clinical database of the Small Animal Teaching Hospital, University of Liverpool, was searched for dogs treated with the DMAC protocol from January 2007 to August 2017. To be eligible for this study, patients had to meet the following inclusion criteria: (1) cytological or histological diagnosis of high-grade cNHL; (2) multicentric disease; (3) assessment of response based on peripheral lymph node size; (4) progressive disease prior to starting the DMAC protocol. Patients who did not receive melphalan (week 2) due to progressive disease and protocol discontinuation after week 1 of the protocol were not excluded. In this study, it was also identified whether patients had relapsed or resistant lymphoma, because different responses could have been seen in these two populations. Relapse was defined as disease progression or recrudescence in a patient that was no longer receiving chemotherapy. Resistance was defined as progressive disease while receiving chemotherapy.24,25 The study was approved by the University of Liverpool ethics committee (VREC549).

## *Medical record review*

Data retrieved from the medical records included standard signalment data (sex, age, breed), weight, body condition score, lymphoma features (type, subtype, stage, substage, immunophenotype, presence of hypercalcaemia), previous chemotherapy (response to first-line protocols , response to previous rescue protocols, number of previous relapses, number of previous chemotherapy agents/protocols received, number of epirubicin doses, toxicity events prior to DMAC initiation), data relating to the DMAC protocol (administration of L-asparaginase within 14 days prior to DMAC initiation, haematological abnormalities prior to DMAC initiation, duration from initial diagnosis to DMAC initiation, actinomycin-D/cytarabine/melphalan/dexamethasone dosage, route of cytarabine administration, modifications in the DMAC protocol throughout treatment, results of complete blood count (CBC) with associated manual differential, documented toxicity, response to DMAC, time from initiation to discontinuation of DMAC protocol (TTD), reason for DMAC discontinuation), rescue therapy after DMAC, overall survival time and cause of death.

## *Diagnosis and staging*

In all cases minimum staging included physical examination, CBC with manual differential count, biochemistry, and lymph node cytology/histology. According to clinician’s discretion and client’s wishes further diagnostics including flow cytometry, immunohistochemistry, urinalysis, thoracic radiographs, abdominal ultrasound, splenic cytology, hepatic cytology, and bone marrow cytology were also performed. In this study dogs were classified according to the World Health Organisation (WHO) five-stage criteria for canine lymphoma only when either hepatosplenic cytology, and/or bone marrow biopsy, and/or cytological confirmation of other organ involvement was performed.26

## *Treatment protocol*

The DMAC protocol is summarised in Table 1. Actinomycin-D (Orphan Europe, Puteaux, France) was administered intravenously with a target dosage of 0.75mg/m2. Cytarabine (Pfizer, Hampshire, UK) was administered subcutaneously as a single dose or intravenously as an infusion over 8 hours with a target dosage of 300mg/m2. Dexamethasone was administered intravenously (Norbrook, Newry, UK) or *per os* (Aspen, Berkshire, UK) with a target dosage of 1mg/kg (calculated to the nearest 2mg when administered *per os).* Melphalan (Aspen, Berkshire, UK) was administered *per os* with a target dosage of 20mg/m2 calculated to the nearest 2mg. The protocol was discontinued in dogs who showed progressive disease during week 1/at day 8 and these dogs did not receive melphalan.

## *Response assessment*

Clinical response to treatment was assessed via lymph node palpation and measurement of diameter in the largest dimension and then perpendicular to that diameter directly with calipers. Thoracic and/or abdominal imaging was repeated to assess response at clinician’s discretion. A complete response (CR) was defined as disappearance of all measurable disease (i.e. lymph nodes palpably normal), a partial response (PR) defined as >50% but <100% reduction in measurable disease, stable disease (SD) was defined as <50% reduction or <25% increase in measurable disease, and progressive disease (PD) was defined as >25% increase in measurable disease or appearance of new lesions.15 As documented measurements were not available in all dogs, dogs that were noted by the attending clinician to have a CR or PR in their clinical records were also classified as responders. To be classified as responders, clinical response had to persist for at least 14 days, dogs who responded to week 1 of the protocol but then progressed in week 2 were classified as having progressive disease. Treatment was continued in patients that had CR or PR. Treatment was discontinued when there was SD, PD, toxicity or other reasons (financial constraints, euthanasia unrelated with lymphoma). Treatment was discontinued when there was SD according to clinician’s discretion: most often, this was because the patient had not received a lomustine-based rescue protocol, so changing to such a protocol offered the potential to achieve remission.

## *Assessment of toxicity*

Toxicity was assessed by reviewing owner history, results of physical examination, and haematology. Haematology was recommended prior to initiation of the protocol and prior to each subsequent chemotherapy administration. Toxicity was retrospectively graded according to the Veterinary Cooperative Oncology Group common terminology criteria for adverse events v1.1.27 For statistical analysis, recurrent toxicities in the same dog were classified according to the highest grade of that toxicity experienced. Neutropenia, thrombocytopenia, anaemia, vomiting, diarrhoea, and anorexia were evaluated. Dosage adjustments, treatment delays, and substitution of melphalan with chlorambucil were made according to clinician’s discretion.

## *Statistical analysis*

Descriptive statistics were calculated, and data were tested for normal distribution and equal variance by means of the Shapiro Wilk test and F-test, respectively.

The overall, CR and PR rates were defined as the number of dogs achieving CR/PR, CR and PR respectively, compared with the total number of dogs treated. The TTD was defined as the time from the initiation of the DMAC protocol until discontinuation due to PD or toxicity.16 Overall survival time (OS) was defined as the time from original diagnosis to death. Dogs that were in clinical remission at the end of the data collection or discontinued the DMAC protocol because of financial constraints or lymphoma-unrelated death were censored. TTD and OS were calculated using the Kaplan-Meier method. As the timing of euthanasia is determined by a combination of clinical factors, clinician and owner judgement, OS was not an endpoint of this study. However, as OS is commonly used in other publications, this was calculated and reported to facilitate comparison between our study population and other studies.

The following predictor variables were used for statistical analysis: weight, age, sex, presence of documented stage V lymphoma, substage, immunophenotype, response to previous chemotherapy protocols, duration of first remission, time from diagnosis to DMAC initiation, number of previous protocols/chemotherapy agents and relapses, occurrence of neutropenia and thrombocytopenia prior to and at the initiation of DMAC, disease progression during first-line protocol, use of L-asparaginase within 14 days prior to DMAC initiation, dexamethasone/melphalan/actinomycin-D/cytarabine dosage (in mg/m2 body surface area and mg/kg), dexamethasone use. The influence of these variables on response (CR/PR vs SD/PD), occurrence of gastrointestinal toxicity (including anorexia, vomiting and diarrhoea), development of grade 3-4 neutropenia and thrombocytopenia and treatment delay was assessed. The influence of grade 3-4 neutropenia and thrombocytopenia and gastrointestinal toxicity on the occurrence of treatment delay was also analysed. For univariable analysis, Fisher’s exact test, *t-*test and Wilcoxon rank sum test were used to analyse the influence of categorical, continuous normal and continuous non-normal variables on the type of response, respectively. Binomial logistical regression was used for multivariable analysis.

Log rank test was used to compare TTD according to the previously mentioned categorical predictor variables and Cox proportional-hazard regression was used for the previously mentioned continuous variables and for multivariable analysis.

Variables significant at 0.2 on bivariate analyses were entered into a multivariate model followed by a backwards stepwise protocol. A minimum of 10 events per factor analysed was required for this factor to be included in multivariable analysis. All analyses were two-sided, and p<0.05 was considered to be significant. All statistical calculations were carried out with the R statistical software version 3.2.0 using the “survival” package. 28,29

**Results**

## *Patients*

One hundred and two client-owned dogs met the inclusion criteria. One hundred cases were defined as resistant and two were defined as relapsed. In order to improve the homogeneity of the data, the two relapsed lymphomas were excluded, and 100 cases were included in the final analysis. The median age was 7.7 years (range 2-14.5 years). The median weight was 27kg (range 4-71.8kg). There were 19 intact males (M), 41 neutered males (MN), 6 intact females (F) and 34 neutered females (FN). The most commonly affected pure breeds included Boxers (8), Border Collies (7), and Bullmastiffs (7).

High-grade/large cell lymphoma was diagnosed on cytology in 57 cases, histopathology in 34 cases, and a combination of both in 9 cases. Thirty six dogs were assigned a WHO stage: 7 (19%) were stage III, 18 (50%) were stage IV, and 11 (31%) were stage V.26 Of all dogs, 77 (77%) were substage a and 23 (23%) were substage b. Immunophenotype was evaluated in 47 dogs: 30 (64%) were B-cell, 16 (34%) were T-cell, and 1 (2%) was B and T-cell. The methods used for evaluating immunophenotype were as follows: 15 flow cytometry, 9 immunocytochemistry, 23 immunohistochemistry. Hypercalcaemia was documented at the time of diagnosis in 11 dogs (11%), immunophenotype was performed in 5/11: 4/5 were T-cell and 1/5 was B-cell. The overall median survival time was 274 days (range 42-2402 days).

## *Previous treatments and toxicities*

Eighty six dogs (86%) had CEOP as a first-line chemotherapy protocol and 14 dogs (14%) had received a combination of vincristine, cyclophosphamide and prednisolone (COP) as first-line. COP was used as a first-line treatment for financial reasons. Seventy six (76%) achieved complete clinical remission and 21 (21%) achieved partial remission. The median duration of first complete remission was 119 days (range 21-722).

The median number of relapses prior to DMAC was 2 (range 1-5). The median number of chemotherapy protocols prior to DMAC was 2 (range 1-5); summarised in Table 2. Patients that relapsed during COP first-line received CEOP or single agent epirubicin. In patients that relapsed after completing a CEOP protocol, reinduction with CEOP was attempted. Patients that relapsed whilst on CEOP/failed CEOP re-induction received either DMAC as rescue protocol or an alternative rescue protocol. In 52 dogs (52%) DMAC was the first rescue protocol after failing CEOP +/- L-asparaginase. In 40 dogs (40%) a lomustine based rescue protocol was used prior to DMAC. In 8 dogs (8%) single agent epirubicin was used as rescue therapy prior to DMAC.

The median number of previous chemotherapy agents was 4 (range 3-8). Seventy-seven dogs (77%) had received less than or equal to 4 doses of epirubicin and 23 dogs (23%) had received more than 4 doses of epirubicin. Twenty-three dogs (23%) had previously received at least one of the drugs in DMAC: nine dogs (9%) had received previous actinomycin-D (median number doses 1, range 1-5), 6 dogs (6%) had received previous cytarabine (all single dose), and 11 dogs (11%) had received previous melphalan (median number doses 1, range 1-2). These drugs were used while dogs were in complete remission and response could therefore not be assessed.

Information on side effects from previous chemotherapy protocols was available in 93 dogs (93%). Fifty-seven (61%) had experienced neutropenia (19% had grade 3-4), five (5%) had experienced thrombocytopenia (2% had grade 3-4), and thirty-seven (39%) had experienced gastrointestinal toxicity the majority of which were grade 1 and 2.

The median duration from the date of diagnosis to the date of starting DMAC was 154 days (range 31-928). Prior to initiation of the DMAC protocol, cytological confirmation of relapse was obtained in 44 dogs (44%) and in remaining dogs was based on physical examination findings. L-asparaginase was administered to 25 dogs (25%) within 14 days prior to DMAC initiation. The median treatment free period prior to DMAC was 13 days (range 1-34).

## *The DMAC treatment protocol*

The median starting dosages of drugs were: actinomycin-D 0.73mg/m2 (range 0.55-1.02), cytarabine 295.2mg/m2 (range 151.5-408.3), melphalan 19.6mg/m2 (range 12.5-38.2), and dexamethasone 24.4mg/m2 (range 8.25-40.84). In mg/kg the median starting dosages were: actinomycin-D 0.024mg/kg (range 0.018-0.045), cytarabine 9.86mg/kg (range 4.73-18.75), melphalan 0.66mg/kg (range 0.36-1.21), and dexamethasone 0.96mg/kg (0.4-1.6). Cytarabine was administered subcutaneously in 99 cases and intravenously as an infusion over 8 hours in 1 case. A modified DMAC protocol was administered to 38 cases, modifications included: change in cycle frequency/dosing interval (9 dogs; the median cycle at which modification occurred was cycle 4, range 0.5-12), replacement of melphalan with chlorambucil (12 dogs; the median cycle at which replacement occurred was cycle 4, range 0.5-9), and omittance or dosage reduction of dexamethasone at the start of the protocol. Dexamethasone was not administered in 8 dogs (8%): 5/8 were already receiving prednisolone and in 3/8 there were concerns of the risk of gastrointestinal haemorrhage. Interestingly, 5 of the 8 dogs that did not receive dexamethasone failed to achieve CR on CEOP.

## *Response to DMAC*

Of the 100 dogs treated, 21 dogs (21%) achieved CR, 14 (14%) achieved PR, and 65 (65%) had SD or PD, for an overall response rate of 35%. None of the factors examined were statistically associated with response. On univariate analysis, there was a trend in the association between response to DMAC and administration of dexamethasone (p=0.16) although it was not statistically significant. Among the eight dogs that did not have dexamethasone, only one responded to DMAC (12.5%), whereas 34 dogs that had dexamethasone responded to DMAC (37%). Due to the low number of cases, this factor could not be analysed in multivariable analysis.

The protocol was discontinued because of disease progression in 84 dogs (84%), stable disease in 6 dogs (6%) and toxicity in 5 dogs (5%). Five dogs (5%) were discontinued due to financial constraints/lost to follow-up. One dog was still in CR at the time of data analysis (1415 days).

The median TTD was 15 days (range 5-952) and the median number of DMAC cycles was 1 (range 0.5-36). On univariate analysis, factors associated with longer TTD were administration of dexamethasone (p=0.01) and complete and partial response (p<0.01). On multivariate analysis, the following factors were significantly associated with longer TTD: response to DMAC (p<0.01), longer time before starting DMAC (p<0.01), and lower number of previous epirubicin doses (p<0.01). The median TTD for complete responders was 62 days (range 28-952) and 32 days (range 20-70 days) for partial responders (Figure 1).

Six complete responders received more than six cycles of DMAC (range 7-36 cycles) and experienced a longer TTD (median 508, range 126-952 days). Discontinuation was due to PD in two dogs, toxicity in one dog, and sustained CR in three dogs.

## *Toxicity*

Baseline haematology prior to initiation of DMAC was available in 98 dogs. Neutropenia was documented in 2 dogs (both grade 1). Thrombocytopenia was documented in 5 dogs (3 grade 1, 1 grade 2, 1 grade 4).

Table 3 shows the toxicity observed during the DMAC protocol. On univariate analysis, several factors were significantly associated with an increased risk of high-grade (grade 3 or 4) neutropenia: lower body weight (p=0.02), lower body surface area (p=0.02), higher actinomycin-D dosage in mg/kg (p=0.03), dexamethasone dosage in mg/m2 (p=0.03), melphalan dosage in mg/kg (p=0.03), and cytarabine dosage in mg/kg (p=0.02). Interestingly, 3/10 (30%) of the dogs weighing less than 10 kg had high-grade neutropenia, whereas only 4/90 (4.4%) dogs weighing more than 10 kg were affected. Due to the low occurrence of high-grade neutropenia, multivariate analysis could not be performed. The median cycle at which neutropenia first occurred was 1 (0.5-5.5). No patients experienced febrile neutropenia.

On univariate analysis, two factors were significantly associated with an increased risk of high-grade (grade 3 or 4) thrombocytopenia: the occurrence of thrombocytopenia prior to DMAC (p=0.04) and thrombocytopenia at the start of DMAC (p<0.01). Conversely to neutropenia, weight was not associated with the occurrence of high-grade thrombocytopenia. Given the small number of events, multivariate analysis could not be performed. The median cycle at which thrombocytopenia occurred was 1 (0.5-8.5). No patients experienced clinical bleeding due to thrombocytopenia.

Diarrhoea was the most common gastrointestinal toxicity. Table 4 shows the breakdown of gastrointestinal toxicities. The median cycle at which gastrointestinal toxicity first occurred was 0.5 (0.5-17). On univariate analysis, several factors were associated with increased risk of gastrointestinal toxicity: longer time from diagnosis to initiation of DMAC (p=0.01), longer duration of first remission (p<0.01) and thrombocytopenia at the initiation of DMAC (p<0.01). On multivariate analysis, only duration of first remission was significantly (p<0.01) associated with the occurrence of gastrointestinal toxicity. Dog that experienced gastrointestinal toxicity had a median duration of first remission of 151 days (range 38-722 days) whereas dogs without gastrointestinal toxicity had a median duration of first remission of 96 days (range 7-408 days). Gastrointestinal toxicity was managed with symptomatic treatment according to clinician preference and severity (treatment included diet change, maropitant, mirtazapine, loperamide, metronidazole, and probiotics).

Thirty-two dogs (34%) had treatment delays due to toxicity. Treatment delays were 2 days in 1 dog, 3-5 days in 9 dogs, 6-8 days in 19 dogs, and 9-15 days in 3 dogs. On univariate analysis, the occurrence of treatment delay was associated with: lower body surface area (p=0.02), lower body weight (p=0.03), response to second rescue treatment (p=0.03), administration of dexamethasone (p=0.04), higher cytarabine dosage mg/kg (p=0.02), greater than grade 1 gastrointestinal toxicity (p<0.01), high-grade thrombocytopenia (p<0.01) and high-grade neutropenia (p<0.01). As expected, the occurrence of treatment delays were associated with: high-grade thrombocytopenia (p<0.001) and gastrointestinal toxicity greater than grade 1 (p=0.02) on multivariate analysis. Due to the small number of cases, high-grade neutropenia was not included in multivariate analysis but, as expected all the dogs with high-grade neutropenia had treatment delays. The median cycle at which treatment delay occurred was 1 (range 0.5-11.5).

Dosage reductions of 5-20% occurred in 13 dogs (13%) at the clinician’s discretion, usually following high-grade haematological toxicity and/or GI toxicity which either resulted in hospitalisation or was not acceptable to the owners.

Hospitalisation was required in 6 dogs (6%). All 6 dogs presented for gastrointestinal toxicity, however, concurrent high-grade afebrile neutropenia was documented in one dog and high-grade thrombocytopenia in another 2 dogs. No dogs died during hospitalisation. One was hospitalised for less than 24 hours, two were hospitalised for 24-48 hours, and three were hospitalised for more than 48 hours. The median cycle at which hospitalisation occurred was 3 (range 0.5-4.5).

In this study 6 cases received more than 6 cycles of DMAC. After cycle 11, intermittent or persistent grade 1 thrombocytopenia was seen in 3/6 patients. In one case this progressed to grade 3 thrombocytopenia and resulted in treatment discontinuation. In the remaining cases the protocol was modified by changing cycle frequency/dosing interval and substituting melphalan with chlorambucil; grade 1 thrombocytopenia was still documented but did not progress.

## *Treatment after DMAC*

After failing DMAC, 86 dogs (86%) received additional rescue protocols. If the patient had not received a lomustine based protocol previously this was used, otherwise the most common protocols were LMP (chlorambucil, methotrexate, prednisolone) or temozolomide (data not shown). The median survival time following DMAC discontinuation was 45 days (range 0-1415).

# **Discussion**

Practising clinicians currently have a range of rescue protocol options when faced with resistant or relapsed high-grade cNHL lymphoma. One option is the DMAC protocol based on the evidence presented in the two published studies.14,16 However, the discrepancy in efficacy reported by these studies complicates clinicians’ ability to make an evidence-based decision. The primary aim, therefore, of this study was to re-evaluate the efficacy and tolerability of the multiagent DMAC protocol in a cohort of UK dogs with resistant high-grade cNHL lymphoma and so to further inform clinical decision making. The secondary aim, was to identify factors associated with response and toxicity when using the DMAC protocol. Although time to progression is recommended for the assessment of treatment response duration, TTD was considered a more objective temporal measure of treatment efficacy for this type of study, as in a rescue setting, toxicity may be as important as tumour progression to justify protocol discontinuation.30 Additionally, progression was only assessed before new drug administration and treatment delays were common in our population, therefore time to progression could have been overestimated.

Results of this study revealed that the efficacy of the DMAC protocol was limited in our population. The overall response rate was 35% (21% CR, 14% PR) with a median TTD of 15 days (62 days CR, 31.5 days PR). This compares unfavourably with Alvarez *et al*’s (2006) report of an overall response rate of 72% (44% CR, 28% PR) with remission duration of 61 days (112 days CR, 44 days PR).16 Conversely, our results are more consistent with those reported by Parsons-Doherty *et al.* (2014). describing an overall response rate of 43% (16% CR, 27% PR) with a PFS of 24 days (63 days CR, 36 days PR).14 However, there are important differences between the populations used in these studies, which may explain these results. In Alvarez *et al.* only 26% (14/54) dogs had received prior doxorubicin, as the main first-line protocol was COAP (cyclophosphamide, vincristine, cytarabine, prednisolone). Therefore, the DMAC protocol may have been more efficacious because of a lack of anthracycline selection for increased expression of the P170-glycoprotein mediating cross-resistance to actinomycin-D.31 This is further supported by the fact that in Alvarez *et al*, previous treatment with doxorubicin was negatively associated with response.16 In current clinical practice, the main first-line protocols are maintenance-free CHOP and COP.32,33 It is important to be aware of this when considering whether to use the DMAC protocol in clinical practice because first-line treatment may influence clinical outcome.

In the present study, only dogs with resistant lymphoma were included in the final data analysis. The distinction between relapsed and resistant lymphoma is inexact and was not made in the previous two DMAC studies; if their populations included a higher number of relapsed (rather than resistant) lymphomas, this may account for the higher response rate compared to the present study.

The differences in assessment of treatment response duration between DMAC rescue papers also confounds comparison: in Alvarez *et al.*, treatment response was assessed by remission duration, but dogs who did not complete both weeks of the protocol due to progressive disease were excluded, potentially portraying a more favourable outcome.16 In the current study, TTD was assessed, and dogs who had progressive disease during week 1 were included, likely contributing to the lower response rate and the low median TTD, and potentially portraying a less favourable outcome. However, this was considered to be more fully representative of the clinical population.

The cytarabine dosage and route of administration differs slightly between studies. Parsons-Doherty *et al.* hypothesised that the lower cytarabine dosage (200mg/m2) used in their study may have contributed to a lower efficacy in comparison to Alvarez *et al*.14 However, in the present study the same dosage of cytarabine (300mg/m2) was used as in Alvarez *et al*.16 In the present study, all but one patient received cytarabine subcutaneously. This route was chosen because it is the predominant route of administration in previous DMAC studies and is more practical in clinical practice.14,16 Alvarez *et al.* found no significant difference in response between subcutaneous or CRI administration.16 However, a pharmacokinetic study of cytarabine in healthy dogs showed that subcutaneous administration, compared to continuous intravenous administration, limits the ability to maintain steady-state concentrations and overall exposure.34 Although the plasma concentration of cytarabine necessary to produce a clinical response in dogs is unknown, rapid elimination may result in the drug being less efficacious when administered subcutaneously. Overall, although cytarabine route was not associated with response in the Alvarez *et al.* study, based on pharmacokinetic data the contribution of a single dose of subcutaneous cytarabine to protocol efficacy may be questionable. Indeed, the efficacy of cytarabine as single agent even used as a continuous rate infusion in naive patients is questionable.35

A potential point of debate is that in the present study, 30 dogs who experienced progressive disease during week 1 of the protocol (actinomycin-D, cytarabine, and dexamethasone) did not receive week 2 of the protocol (melphalan and dexamethasone). While this contributed to the low median TTD, the clinical decision not to administer melphalan in these cases is supported by evidence for the poor efficacy of melphalan in relapsed canine lymphoma. Mastromauro *et al*. reported only partial responses to single-agent melphalan (0% CR, 16% PR, 16% SD) and time to progression for responders only 14-34 days.36 This justifies switching patients that have not achieved measurable response after DMAC week 1 to other rescue chemotherapy protocols.

A second point of discussion is that 8 cases did not receive dexamethasone. These cases were not excluded because the reasons given for withholding dexamethasone: risk of gastrointestinal haemorrhage and concurrent prednisolone therapy, were considered valid and likely applicable to other patient populations being started on DMAC. However, it is important to consider whether their inclusion impacted the response rates in this study. On univariate analysis, dexamethasone administration was associated with longer TTD (p=0.013) and had a borderline association with prolonged response (p=0.16). Due to low case numbers, inclusion in multivariate analysis was not possible, but these findings may suggest that administration of dexamethasone improves response. However, when analysing the dogs that did not receive dexamethasone, 5/8 did not achieve complete remission on CEOP first-line, suggesting a more aggressive behaviour and this may have driven the lack of response to DMAC, not the absence of dexamethasone.

A further point of discussion is that 23% of dogs had previously received at least one of the DMAC drugs. Therefore, there is the potential for pre-existing drug resistance, which may have reduced overall response rate. However, the number of previous doses of each drug was low (median number doses 1 for all drugs) potentially reducing the selection pressure.

The second aim of this study, was to identify factors associated with response. None of the factors analysed were significantly associated with response. Immunophenotype is an established prognostic indicator, but it was not significantly associated with response or TTD to the DMAC protocol. This is consistent with previous evaluations of the protocol.14,16 Potentially, different histotypes (which were poorly characterised in this study) may have driven response making immunophenotype less relevant. However, given that the predominant histotypes are likely to be diffuse large B-cell lymphoma (DLBCL) and peripheral T-cell lymphoma not otherwise specified (PTCL-NOS) this seems unlikely.1 A more plausible, albeit hypothetical, explanation is that immunophenotype may play a minimal role in the response to rescue protocols in heavily pre-treated lymphomas where acquired resistance mechanisms are more relevant than phenotype.

In the current study, toxicity was relatively frequent, with higher rates of low-grade neutropenia and low-grade gastrointestinal toxicity than previously reported. Overall, toxicity infrequently led to hospitalisation, discontinuation of treatment, or dosage reductions. Similar to previous studies, thrombocytopenia was the most common adverse event. The median cycle at which thrombocytopenia occurred was cycle 1 (range 0.5-8.5), but in the small-subset of patients who received long-term DMAC (>6 cycles) grade 1 thrombocytopenia was common. The reason for thrombocytopenia early on in the course of treatment is unclear. Delayed thrombocytopenia is a commonly reported adverse event following single-agent melphalan, most often occurring 3-4 weeks after drug administration and/or following repeated doses of melphalan; thus likely accounting for low-grade thrombocytopenia in patients on long-term DMAC.31 Early thrombocytopenia is less easily explained; in studies of single agent actinomycin-D in canine lymphoma, thrombocytopenia was rare.37,38 Cytarabine may play a role as other multiagent rescue protocols involving cytarabine are associated with high rates of thrombocytopenia: in protocols combining bleomycin and cytarabine, or carboplatin and cytarabine, thrombocytopenia occurred in 40% (8/20) and 94% (16/17) respectively.12,39 However, when interpreting multiagent protocols it is difficult to determine contribution of individual drugs. Moreover, the more advanced course of disease and heavy pre-treatment in these cases may confound the ability to differentiate acute chemotherapy toxicity from disease progression or cumulative chemotherapy toxicity. In the present study, the occurrence of gastrointestinal toxicity was associated with a longer duration of first remission. This may suggest that the risk of gastrointestinal toxicity increases with the duration of previous chemotherapy treatment. However, this finding could also be incidental due to the relatively small population and should be confirmed in a larger cohort of dogs.

In the current study, neutropenia was less common, and the majority were low-grade in comparison to previous studies. Multivariate analysis could not be performed due to low occurrence of high-grade neutropenia. However, an interesting finding was that high-grade neutropenia was more common in dogs weighing less than 10kg. Moreover, on univariate analysis, dosages of cytarabine, melphalan, and actinomycin-D were associated with high-grade neutropenia when expressed in mg/kg, but not in mg/m2. Therefore, there is the potential that smaller dogs might be relatively overdosed when using body surface area, as previously described for melphalan, lomustine, procarbazine and doxorubicin.40-42

Gastrointestinal toxicity was less common than haematological, and the majority were low-grade. The median cycle at which gastrointestinal toxicity occurred was cycle 0.5 (after the administration of actinomycin-D and cytarabine), in comparison to haematological toxicity which occurred at cycle 1 (after the administration of melphalan). As the current study is retrospective, gastrointestinal toxicity is likely to have been underestimated, particularly if low-grade, as it may have not been reported by the owners or noted in the clinical records.

In Alvarez *et al*., the DMAC protocol was discontinued after 5-8 cycles for complete responders. In the current study a small subset of patients was successfully managed on long-term DMAC, receiving more than 6 cycles. Most had prolongations in cycle duration, and chlorambucil was substituted for melphalan to minimise haematological toxicity. Despite this, grade 1 thrombocytopenia was common, but rarely necessitated treatment discontinuation. It seems reasonable to recommend continuing beyond the 8 cycles in patients still in remission.

The main limitation of this study is its retrospective nature. Despite this study having a larger cohort of dogs than the previous evaluations of DMAC, it is still a relatively small sample, limiting the statistical power. Initial staging was variable and dogs were not consistently re-staged prior to receiving DMAC or during the follow-up period. The exclusion of cases whose response could not be assessed on peripheral lymph node involvement was performed to try to minimise overestimating CR in cases that would otherwise rely on diagnostic imaging assessment, but may have created bias.

In conclusion, in this study, response to DMAC was lower and of generally shorter duration than previously reported. Toxicity was frequent, but rarely led to hospitalisation or discontinuation of treatment. Compared to previous studies of the DMAC protocol, the population in this study was more heavily pre-treated, which may have contributed to the less favourable outcome. However, as this is a common scenario faced by clinicians managing resistant canine lymphoma it may be more representative of current populations.

**Conflict of interest**

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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**Figure Legend**

**Figure 1.** Time from initiation to discontinuation for dogs who achieved CR (62 days, range 28-952), PR (32 days, range 20-70), and SD/PD (13 days, range 6-71) treated with DMAC (p<0.001).