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Peripheral blood lymphocyte/monocyte ratio as a useful prognostic factor in dogs with diffuse large B-cell lymphoma receiving chemoimmunotherapy

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Highlights

- The majority of dogs with diffuse large B-cell lymphoma (DLBCL) relapse and die as a consequence of the disease
- New prognostic models are needed to characterize dogs with a poor prognosis
- A lymphocyte/monocyte ratio (LMR) ≤ 1.2 was associated with inferior time-to-progression and lymphoma specific survival
- LMR at diagnosis is a prognostic indicator for dogs with DLBCL receiving chemoimmunotherapy

Abstract

Diffuse large B-cell lymphoma (DLBCL) is the most frequent canine lymphoid neoplasm. Despite treatment, the majority of dogs with DLBCL experience tumour relapse and consequently die, so practical models to characterize dogs with a poor prognosis are needed. This study examined whether the lymphocyte/monocyte ratio (LMR) can predict outcome in dogs with newly diagnosed DLBCL with regards to time-to-progression (TTP) and lymphoma specific survival (LSS).

A retrospective study analyzed the prognostic significance of LMR obtained at diagnosis by flow cytometry (based on morphological properties and CD45 expression) in 51 dogs that underwent complete staging and received the same treatment, comprising multi-agent chemotherapy and administration of an autologous vaccine. Dogs with an LMR ≤ 1.2 (30% of all cases) were found to have significantly shorter TTP and LSS, and it was concluded that LMR was a useful independent prognostic indicator with biological relevance in dogs with DLBCL treated with chemoimmunotherapy.

Keywords: Chemoimmunotherapy; Diffuse large B-cell lymphoma; Dog; Lymphocyte-monocyte ratio; Prognosis

Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most frequent canine lymphoid neoplasm (Ponce et al., 2010; Villa et al., 2011; Aresu et al., 2013). In spite of important advances in treatment, a significant proportion of dogs develop tumour relapse or refractory disease after initial remission, and many die as a result (Marconato et al., 2011a; Aresu et al., 2013).

Several factors assessed at diagnosis have been proposed as predictors of clinical outcome in dogs with DLBCL, including stage, substage (Marconato et al., 2013) and, more recently, gene expression profiling (Richards et al., 2013) and copy number aberrations (Aricò et al., 2014). Additional prognostic models that are inexpensive, widely available, practical, and easily interpreted by clinicians are needed to characterize dogs with a poor prognosis and to facilitate treatment.

There is increasing interest in the role of lymphocytes and monocytes in the pathogenesis of human cancer (Bruckner et al., 1982; Koh et al., 2012; Wilcox et al., 2012; Batty et al., 2013; Li et al., 2013). In particular, lymphocytes are considered to reflect the host immune homeostasis, while monocytes are regarded as surrogate markers of the tumour microenvironment (Ciocca et al., 2007; Gabrilovich et al., 2009; Calderwood et al., 2012).

It has been shown that the absolute lymphocyte count (ALC) and absolute monocyte count (AMC) at diagnosis has prognostic significance in dogs with osteosarcoma by predicting disease-free interval (DFI) (Sottnik et al., 2010). In a retrospective study of 26 dogs with lymphoma, increased AMC and neutrophil count at diagnosis were significantly associated with a decrease in DFI (Perry et al., 2011). More recently, the ALC and

neutrophil/lymphocyte ratio were evaluated retrospectively in 77 dogs with multicentric lymphoma but no prognostic relevance was documented (Mutz et al., 2013).

Novel promising cancer therapies, such as the use of vaccines, represent one of the most exciting opportunities in human and veterinary oncology (Marconato et al., 2015). Active immunotherapy directs the protective capacity of the immune system towards eliminating malignant cells, and establishes a long-lasting anti-tumour immunity through the capacity to exhibit memory. Recently, immunotherapy combined with chemotherapy has been demonstrated to prolong both time-to-progression (TTP) and lymphoma specific survival (LSS) in dogs with DLBCL (Marconato et al, 2014). However, a possible disadvantage of active immunotherapy is its reliance on the patient's immune system, which may be compromised or deregulated by the tumour itself. It is important to identify baseline biomarkers that can be used to predict treatment response so allowing patients to be stratified into treatment groups.

In humans with DLBCL, the ALC/AMC ratio (LMR) at diagnosis has been reported to be a prognostic factor for clinical outcome regardless of the International Prognostic Index (Rambaldi et al., 2013; Li et al., 2014). We hypothesized that LMR may also be used to predict prognosis in dogs with newly diagnosed DLBCL. This retrospective study was designed to evaluate the prognostic value of LMR obtained at diagnosis by flow cytometry in a large cohort of canine DLBCL cases treated with chemoimmunotherapy at a single institution in Italy.

Material and methods

Eligibility

Dogs were considered eligible if they had a histological and immunohistochemical diagnosis of DLBCL according to the criteria established by the World Health Organization (WHO) classification (Valli et al., 2011). Dogs that had received previous treatment for their lymphoma (including glucocorticoids within the last month) were excluded.

For all dogs, staging work-up consisted of history, physical examination, haematology (complete blood count [CBC], blood smear evaluation), serum biochemistry (including lactate dehydrogenase [LDH]), thoracic radiographs, abdominal ultrasound, as well as cytological and flow cytometric evaluation of a fine-needle aspirate of an enlarged peripheral lymph node, of peripheral blood (PB) and of a bone marrow (BM) aspirate (Marconato et al., 2011b).

CBC was performed on the day of diagnosis using an automated laser haematology analyser (ADVIA 120 with multispecies software for veterinary use, Siemens Healthcare Diagnostics) on whole PB samples collected in 2.5 mL K_3 ethylene diamine tetraacetic acid (EDTA) tubes. Lymphocyte and monocyte percentages were calculated based on flow cytometric (FC) analysis, by taking into account morphological scatter grams, CD45 (CD45-APC; clone YKIX716.13, pan-leucocyte marker; Abad Serotec) and CD21 (clone CA2.1D6, B cell marker; AbD Serotec) expression (Comazzi et al, 2006). In particular, small cells with low complexity and high/intermediate CD45 expression were classified as lymphocytes, whereas CD21-negative large cells with intermediate complexity and high CD45 expression were classified as monocytes. ALC and AML were calculated based on the white blood cells counts generated by the laser analyser, and the percentages of lymphocytes and monocytes obtained from FC analysis. LMR was calculated as the ratio of absolute counts between peripheral lymphocytes and monocytes.

Treatment and response assessment

With prior written informed consent by the owners, all dogs were treated with a CHOP (cyclophosphamide, vincristine, doxorubicin, prednisone)-based protocol with the incorporation of Apavac immunotherapy, as previously described (Marconato et al., 2014). Briefly, chemotherapy consisted of L-asparaginase (Leunase, Rhône-Poulenc Rorer; week 1), vincristine (Vincristina, Teva; weeks 2, 3, 4 and 13), cyclophosphamide (Endoxan, Baxter; weeks 2 and 13), doxorubicin (Adriblastina, Pfizer; weeks 7 and 16), lomustine (Cecenu, Medico; weeks 10 and 19), and prednisone (Vetsolone, Bayer; weeks 1 to 20). Apavac, consisting of hydroxyapatite ceramic powder and heat shock proteins purified from the dogs' tumours, was injected into the dermis on weeks 4, 5, 6, 7, 12, 16, 20 and 24.

Response to treatment was evaluated at each treatment session according to previously published criteria (Vail et al., 2010). Responses were required to last for at least 28 days. End-staging was carried out at the end of treatment, and every clinical, radiological, ultrasonographic or laboratory investigation that disclosed abnormalities at pre-treatment staging was repeated. PB and BM were sampled again for FC analysis in all cases. Afterwards, all dogs were followed-up on a monthly basis for the first year and then every 2 months. Rescue treatment was offered to the owners in case of relapse.

Statistical analysis

Univariate Cox's proportional hazard regression analysis was performed to determine a possible association between selected variables and TTP and LSS, respectively. Variables with $P \leq 0.3$ upon univariate analysis were then included in a backward elimination multivariate analysis. For categorical variables, Kaplan-Meier curves were drawn and compared by log-rank test. TTP was calculated from the start of treatment to disease

progression (Vail et al., 2010). Dogs lost to follow-up or that died for causes unrelated to lymphoma and before disease progression, as well as those still in complete remission (CR) at the end of the study, were censored for TTP analysis. LSS was measured as the interval between the start of treatment and death due to lymphoma (Vail et al., 2010). Dogs alive at the end of the study, lost to follow-up or that died due to causes other than lymphoma were censored for LSS analysis.

Variables assessed for prognostic significance for TTP and LSS included: breed (pure or mixed), age (< or ≥ 10 years), sex (male or female), weight (< or ≥ 10 kg), clinical stage (I-V), substage (a or b), involvement of extranodal sites (yes or no), LDH (within or outside the reference interval; reference interval: 0-170 IU/L), evidence of BM involvement by FC analysis (presence or absence), evidence of PB involvement by FC analysis (presence or absence), ALC at presentation, AMC at presentation, and LMR. LMR could not be included as a continuous variable in the Cox's analysis, because of a violation of the proportional hazards assumption (PHA). Thus, it was dichotomized based on different arbitrary cut-offs equal to the 10th, 20th, 25th, 30th, 40th and 50th percentiles, respectively. The cut-off showing the lowest *P* value with the univariate analysis was then included in the multivariate analysis and used to draw Kaplan-Meier curves. Statistical analysis was performed using IBM Statistical Package for Social Science (SPSS) v.17.0 for Windows. Significance was set at $P \leq 0.05$ for all tests.

Results

Dog population

Fifty-one dogs were enrolled. The dogs represented 22 breeds, including mixed-breed dogs (n = 12), Rottweiler (n = 5), Dobermann (n = 4), German Shepherd (n = 3), Cocker

spaniel (*n* = 3), Golden retriever (*n* = 3) and 16 other breeds each represented by one to two animals. Median age was 8 years (range, 4-15 years), and median weight was 27 kg (range, 5-60 kg). Twenty-five (49%) were females (of which 17 were spayed), and 26 (51%) dogs were males (of which 7 were castrated).

Based on the WHO staging, one (2%) dog had stage Ia disease, four (7.8%) dogs had stage IIIa disease, 30 (58.8%) dogs had stage IV disease (24 with substage a, six had substage b), and 16 (31.4%) dogs had stage V disease (eight with substage a, eight with substage b). Among dogs with stage V disease, eight (50%) dogs had PB and BM involvement, five (31.3%) dogs had BM involvement, one (6.3%) dog had PB involvement, one (6.3%) dog had pulmonary involvement, and one (6.3%) dog had BM and skin involvement. Twenty-eight (54.9%) dogs had increased LDH activity.

Median ALC was 967.5/μL (range, 156-6429/μL), median AMC was 565.6/μL (range, 126-4392.8/μL), and median LMR was 1.76 (range, 0.09-10.32).

Outcome

Overall, 41 (80.4%) dogs achieved CR, six (11.8%) had PR, two (3.9%) had a SD and one (1.9%) dog's disease progressed after 13 days. In one dog, the first treatment response could not be established, because its follow-up was shorter than 28 days.

Overall median TTP was 217 days (range, 13-649 days). Thirty-eight (74.5%) dogs progressed during the study period, with a median TTP of 192 days (range, 13-649 days), whereas 13 (25.5%) dogs never relapsed. Among these, seven died for causes unrelated to lymphoma after 15, 42, 138, 155, 291 342 and 450 days, respectively, and were still in CR for

lymphoma. The remaining six dogs were still alive in CR at data analysis closure, with a median follow-up of 162 days (range, 54-528 days).

Univariate Cox's proportional hazard regression analysis revealed substage b (P = 0.009), LDH activity (P = 0.038) and AMC (P = 0.02) exhibiting significant association with TTP. Kaplan-Meier curves, and log-rank test confirmed the significant influence of substage and LDH activity on TTP (P = 0.007 and P = 0.033, respectively). Regarding LMR, significant influence on TTP was detected for the 10th (P = 0.038), 20th (P = 0.003), 25th (P = 0.009), and 30th percentiles (P = 0.001). The 30th percentile was equal to 1.2 and this value was used as cut-off for further analyses. Kaplan-Meier curves for LMR are depicted in Fig.1.

Upon multivariate analysis, substage (P = 0.018), LDH activity (P = 0.049), AMC (P = 0.032), and LMR $\leq$ or $>$ 1.2 (P = 0.008) were independently associated with TTP. In particular, the probability of progressing was more than two-fold higher in symptomatic dogs or in dogs with elevated LDH activity compared with their respective counterparts, whereas it was more than three-fold higher in dogs with LMR $\leq$ 1.2 compared with dogs with LMR $>$ 1.2. Specific TTP for significant variables are listed in Table 1.

Overall median LSS was 413 days (range, 15-1090 days). Thirty-one (60.8%) dogs died from their lymphoma during the study period, with a median LSS of 298 days (range, 54-767 days); 10 (19.6%) dogs died for unrelated causes after 15, 42, 114, 138, 155, 211, 291, 342, 450 and 845 days, respectively; and 10 (19.6%) dogs were still alive at data analysis closure, with a median follow-up of 164 days (range, 44-1090 days).

Based on univariate Cox's proportional hazard regression analysis, LSS was influenced by age (P = 0.030), substage (P = 0.014), and AMC (P = 0.001). Kaplan-Meier curves and log-rank test confirmed the statistically significant influence of age and substage on LSS (P = 0.025 and P = 0.011, respectively). LDH activity was almost statistically significant at univariate Cox's proportional hazard regression analysis (P = 0.051) and significant using the log-rank test (P = 0.046). Regarding LMR, statistically significant influence on LSS was detected for the 10th (P = 0.022), 20th (P = 0.004), 25th (P = 0.005) and 30th percentiles (P = 0.002). Thus, 1.2 (30th percentile) was used as the LMR cut-off for further analysis. Kaplan-Meier curves of LMR are depicted in Fig.1.

Based on multivariate analysis, sex (P = 0.048), age (P = 0.018), substage (P = 0.034), PB infiltration (P = 0.031), AMC (P = 0.002) and LMR ≤ or > 1.2 (P = 0.003) were independently associated with LSS. In particular, the probability of death due to lymphoma was more than two-fold higher in dogs < 10 years old, in symptomatic dogs, and in dogs with elevated LDH activity, compared with their respective counterparts, whereas it was more than four-fold higher in dogs with LMR ≤ 1.2 compared with dogs with LMR > 1.2. Specific LSS for significant variables are listed in Table 2.

Discussion

In the present study, we evaluated whether LMR at diagnosis is a good prognostic indicator in dogs with DLBCL treated with the same first-line chemoimmunotherapy protocol, thereby identifying high-risk patients. To our knowledge, this is the first report demonstrating that a decreased LMR at diagnosis is associated with inferior TTP and LSS in dogs with newly diagnosed DLBCL.

In contrast to conventional prognostic variables, LMR does not incorporate patient and tumour characteristics, which emphasises the simplicity of this index because it is formed by FC data related to a patient's adaptive immune response. Unfortunately, LMR could not be included as such in the Cox's analysis as it did not satisfy the test's requirements for inclusion of variables. Thus, we could not investigate whether a reduction in the duration of TTP or LSS was proportional to a decrease in LMR. Consequently, we subdivided dogs into groups based on arbitrarily selected LMR cut-offs, using percentiles as a track, and selected the cut-off value that best discriminated between two prognostic groups.

To differentiate between atypical lymphocytes and monocytes and to reduce the uncertainty of the results obtained from the manual leukocyte differential count, FC analysis was used thereby eliminating the risk of leukocyte misclassification mostly in those cases with marked PB infiltration (approximately 18% of cases). Monocytes are easily recognized by the expression of CD14, but unfortunately this marker was not available for all samples. Thus, the identification of monocytes was based on their scatter properties and CD45 expression.

We evaluated the prognostic impact of the ALC, AMC, and LMR at diagnosis on the outcomes of dogs with DLBCL treated with chemoimmunotherapy. Based on our findings, LMR obtained by FC analysis provided prognostic information independent of the already described prognostic variables, including clinical stage, substage, and LDH (Zanatta et al., 2003). Indeed, a LMR value of ≤1.2 at diagnosis in dogs with DLBCL was significantly associated with shorter TTP and LSS, suggesting that lymphocytes and monocytes may play a role in dogs with DLBCL receiving chemoimmunotherapy. This cut-off was equal to the 30th

279 percentile of LMR, meaning that 30% of the dogs in the study had an LMR value that was
280 lower than or equal to the selected cut-off, indicating a worse prognosis.
281
282 As anticipated, a low LMR may be the result of a combination of monocytosis and
283 lymphopenia, reflecting defective immunity to tumour cells, and the finding that LMR acts as
284 prognostic indicator can be explained by the following mechanisms. Firstly, lymphoma B-
285 cells can recruit monocytes to support the survival and proliferation of neoplastic B cells and
286 suppress the proliferation of normal T cells (Lin et al., 2011). Secondly, monocytes contribute
287 to the suppression of host anti-tumour immunity and play an important role in tumour
288 angiogenesis, which in turn promotes tumour growth (Gabrilovich et al., 2009). Thirdly,
289 tissue-associated macrophages are a source of vascular endothelial growth factor (VEGF) and
290 matrix metalloproteinase 9 (MMP-9), which both promote tumour angiogenesis (Aricò et al.,
291 2013). Altogether, it is not surprising that monocytosis is considered as an adverse prognostic
292 factor in various tumours (Schmidt et al., 2005; Sottnik et al., 2010; Perry et al., 2011).
293 Conversely, lymphopenia may be considered as a surrogate marker of host immunological
294 incompetence (Shivakumar et al., 2006) since lymphocytes (particularly T cells) are important
295 mediators of cellular immune responses and may be required for vaccine-mediated destruction
296 of malignant B cells (Ciocca et al., 2007; Calderwood et al., 2012; Marconato et al., 2014).
297
298 The dogs in our series received an autologous vaccine in addition to dose-intense
299 chemotherapy. A low LMR may impact the efficacy of immunotherapy by a simple reduction
300 of the circulating effector cells, since the immune system probably mediates the effect of the
301 therapeutic vaccine used here. Hence, these results may help clinicians select dogs that are
302 more prone to respond to chemoimmunotherapy. Since LMR reflects both host immunity and
303 the tumour microenvironment, it may be superior for predicting the response to

chemoimmunotherapy compared with lymphocytes or monocytes alone, which only partially reflect cancer-related immunity.

The results of this study should be interpreted with caution because of its limitations, including the small population size with a relatively short median follow-up time. Despite the homogeneous staging work-up and the standardized therapeutic protocol, it was not possible to provide molecular data to define the heterogeneity of the tumours included in the study, such as the definition of the DLBCL molecular profile and chromosomal aberrations (Richards et al., 2013; Aricò et al., 2014). It is possible that distinct molecular signatures may have an influence on the immunological response not only in humans but also in dogs. Indeed, human DLBCLs originating from activated B cell-like tumour cells are associated with a poorer prognosis and a more severe alteration of the immune system when compared with DLBCLs originating from the germinal centre tumour cells (Mehta-Shah and Younes, 2015). Finally, due to the retrospective nature of the study, CD5 (a T-cell marker) and CD14 (a monocyte marker) were not included in the FC panel, and the percentages of lymphocytes and monocytes were obtained only by combining morphological properties and CD45 expression. However, this approach has already been described in the literature, and adequately identified leukocyte subclasses in dogs (Comazzi et al., 2006).

Conclusions

The prognosis of DLBCL in dogs may be associated with the role of host immunity and the tumour microenvironment, which are not taken into consideration by clinical staging. Easy-to-measure laboratory parameters reflecting host immunity and the tumour microenvironment may therefore be necessary in order to have a robust prognostic measure for canine DLBCL. LMR determined by FC analysis may represent a useful additional tool

for predicting the prognosis of dogs with DLBCL. This was found to be the case in dogs receiving chemoimmunotherapy, and further studies with dogs treated with traditional chemotherapeutic protocols are required.

Conflict of interest statement

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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Table 1

Time-to-progression (TTP) for 51 dogs with diffuse large B-cell lymphoma (DLBCL) receiving chemoimmunotherapy according to specific variables.

Variables (number of dogs)	Median TTP in days (range)	Univariate analysis, *P*	Multivariate analysis, *P*	Log-rank test, *P*	HR (95% CI)[a]
Substage:					
- a (*n*=37)	241 (13-649)	0.009*	0.018*	0.007*	2.650 (1.273-5.515)
- b (*n*=14)	117 (33-312)				
LDH activity:					
- normal (*n*=23)	240 (41-649)	0.038*	0.049*	0.033*	-
- increased (*n*=28)	122 (13-493)				2.033 (1.038-3.981)
LMR					
- ≤ 1.2 (*n*=15)	117 (41-1019)	0.001*	0.008*	0.000*	3.691 (1.706-7.985)
- > 1.2 (*n*=36)	251 (21-588)				

[a] HR, hazard ratio; 95% CI, 95% confidential interval

* $P \leq 0.05$

Table 2

Lymphoma specific survival (LSS) for 51 dogs with diffuse large B-cell lymphoma (DLBCL) according to specific variables.

Variables (number of dogs)	Median LSS in days (range)	Univariate analysis, *P*	Multivariate analysis, *P*	Log-rank test, *P*	HR (95% CI)[a]
Sex:					
- female (*n*=25)	361 (54-666)	0.282	0.048*	0.278	1.158 (0.604-2.220)
- male (*n*=26)	480 (15-1090)				
Age:					
- < 10 years (*n*=30)	480 (15-1090)	0.030*	0.018*	0.025*	2.363 (1.087-5.137)
- ≥ 10 years (*n*=21)	361 (42-565)				
Substage:					
- a (*n*=37)	470 (42-1020)	0.014*	0.034*	0.011*	2.769 (1.229-6.240)
- b (*n*=14)	188 (22-530)				
LDH activity:					
- normal (*n*=23)	470 (42-1090)	0.051	0.562	0.046*	2.102 (0.995-4.440)
- increased (*n*=28)	361 (15-623)				
PB infiltration					
- absent (*n*=38)	442 (15-1090)	0.248	0.031*	0.243	1.583 (0.726-3.454)
- present (*n*=13)	361 (93-585)				
LMR:					
- ≤ 1.2 (*n*=15)	188 (54-442)	0.002*	0.003*	0.001*	4.131 (1.719-9.931)
- > 1.2 (*n*=36)	480 (15-1090)				

HR, hazard ratio; 95% CI, 95% confidential interval; PB, peripheral blood

* $P \leq 0.05$

492 Figure legend

493 Fig. 1. Kaplan-Meier curves representing time-to-progression (TTP) and lymphoma specific
494 survival (LSS) for 51 dogs with diffuse large B-cell lymphoma (DLBCL) with a lymphocyte/
495 monocyte ratio (LMR) ≤ 1.20 (continuous line) and > 1.20 (dotted line) assessed by flow
496 cytometry. + = censored case

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