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Enhanced therapeutic effect of APAVAC immunotherapy in combination with dose-intense chemotherapy in dogs with advanced indolent B-cell lymphoma

L. Marconato[a,*], D. Stefanello[b], S. Sabattini[c], S. Comazzi[b], F. Riondato[d], P. Laganga[a], P. Frayssinet[e], S. Pizzoni[a], N. Rouquet[e], L. Aresu[f]

[a] Centro Oncologico Veterinario, Sasso Marconi, BO, Italy
[b] Department of Veterinary Sciences and Public Health, University of Milan, Milan, Italy
[c] Department of Veterinary Medical Sciences, University of Bologna, Bologna, Italy
[d] Department of Veterinary Sciences, University of Turin, Turin, Italy
[e] Urodelia, St Lys, France
[f] Department of Comparative Biomedicine and Food Science, University of Padova, Agripolis Legnaro, Italy

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ABSTRACT

The aim of this non-randomized controlled trial was to compare time to progression (TTP), lymphoma-specific survival (LSS), and safety of an autologous vaccine (consisting of hydroxyapatite ceramic powder and Heat Shock Proteins purified from the dogs' tumors, HSPPCs-HA) plus chemotherapy versus chemotherapy alone in dogs with newly diagnosed, clinically advanced, histologically confirmed, multicentric indolent B-cell lymphoma. The vaccine was prepared from dogs' resected lymph nodes and administered as an intradermal injection. Forty-five client-owned dogs were enrolled: 20 dogs were treated with dose-intense chemotherapy, and 25 received concurrent immunotherapy. Both treatment arms were well tolerated, with no exacerbated toxicity in dogs also receiving the vaccine. TTP was significantly longer for dogs treated with chemo-immunotherapy versus those receiving chemotherapy only (median, 209 versus 85 days, respectively, $P = 0.015$). LSS was not significantly different between groups: dogs treated with chemo-immunotherapy had a median survival of 349 days, and those treated with chemotherapy only had a median survival of 200 days ($P = 0.173$). Among vaccinated dogs, those mounting an immune response had a significantly longer TTP and LSS than those with no detectable response ($P = 0.012$ and $P = 0.003$, respectively). Collectively these results demonstrate that vaccination with HSPPCs-HA may produce clinical benefits with no increased toxicity, thereby providing a strategy for enhancing chemotherapy in dogs with advanced indolent lymphoma.

1. Introduction

In humans and dogs, indolent lymphomas represent a group of incurable slow-growing tumors, characterized by a continuous relapse pattern, for which there are no defined first-line therapies [1]. The biologic behavior is extremely variable, with some patients having an aggressive course and death within a few months despite intense treatment, and others living for years, never requiring therapy [2–7].

While in most cases indolent lymphoma is discovered incidentally and harbors a good prognosis [5–7], emerging data support that some dogs experience pathological and clinical progression [2].

A universally accepted definition of "advanced" lymphoma does not exist; nevertheless, it is reasonable to reserve the term

Abbreviations: BM, bone marrow; B-SLL, B-cell small lymphocytic lymphoma; CR, complete remission; DLBCL, diffuse large B-cell lymphoma; DTH, delayed-type hypersensitivity; FC, flow cytometry; FL, follicular lymphoma; HSP, heat shock protein; HSPPC, heat shock protein-peptide complex; LDH, lactate dehydrogenase; LN, lymph node; LSS, lymphoma-specific survival; MRD, minimal residual disease; MZL, marginal zone lymphoma; PARR, polymerase chain reaction for antigen receptor rearrangement; PB, peripheral blood; PCV, packed cell volume; PD, progressive disease; PLT, platelets; PR, partial remission; TTP, time to progression; VCOG-CTCAE, Veterinary Co-operative Oncology Group – Common Terminology Criteria for Adverse Events; WHO, World Health Organization.

* Corresponding author at: Centro Oncologico Veterinario, via San Lorenzo 1/4, 40037 Sasso Marconi, BO, Italy. Tel.: +39 0516751871.
E-mail address: marconato@centroncologicovet.it (L. Marconato).

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"advanced" for stage IV–V patients, with disease bulk or symptoms [2]. With this premise in mind, it comes as no surprise that the treatment of this disease is controversial. As a rule, an advanced WHO clinical stage (IV–V) and the presence of symptoms typically speak in favor of undertaking treatment. Although chemotherapy is believed to improve remission duration and survival, the disease is essentially incurable [3].

Cancer therapeutic vaccines are a promising yet challenging strategy. The past two decades have witnessed approaches to incorporate active immunotherapy into the multimodal care of veterinary oncology patients for whom there continues to be unmet medical need [8–14].

Tumor-derived HSPPC coupled with hydroxyapatite has recently proven to induce immunity against autologous tumors in a clinical trial on canine DLBCL, ultimately translating into clinical efficacy [13].

A component of HSPPC, HSP, functions as protein chaperone, aimed at stabilizing its associated molecules when the cells undergo stress situations. Cancer cells synthesize high amounts of HSPs [15]. The immune response induced by an HSP-based vaccine begins with antigen-presenting cells taking up antigenic epitopes with HSP and presenting them on major histocompatibility complex class-I molecules [15–17]. Presented epitopes are then recognized by CD8 T-cells and activate the cellular immune response.

Thus, by purifying the cancer HSPs and by associating these molecules to an adjuvant, a vaccine against specific antigens of the tumor cells is obtained [13,15–17]. Hydroxyapatite shows adjuvant properties, and HSPs have an affinity for its surface, thereby facilitating purification using hydroxyapatite powder columns [18].

Based on the encouraging results obtained in dogs with DLBCL, a clinical trial was carried out to evaluate the efficacy and safety of autologous HSPPC-vaccine in combination with chemotherapy as the primary treatment for dogs with newly diagnosed, advanced indolent B-cell lymphoma.

2. Materials and methods

2.1. Study design

This study was designed as a prospective, controlled, non-randomized, bi-center trial to investigate TTP and LSS (primary endpoints) and safety (secondary endpoint) of chemotherapy (Group 1) in comparison with chemo-immunotherapy (Group 2).

2.2. Inclusion criteria

Dogs were recruited at the Centro Oncologico Veterinario and the Department of Veterinary Science and Public Health (University of Milan) between 2011 and 2014. To be eligible for recruitment, dogs were required to have untreated, histologically confirmed, indolent B-cell lymphoma (i.e., FL, B-SLL, or MZL) of advanced (IV–V) clinical stage.

Included dogs were required to undergo a complete staging work-up [19,20]; Supplemental Data]. The cut-off for PB and BM infiltration was set at >0.9% of medium/large CD21+ cells recognized as neoplastic because of larger size and/or different fluorescence intensity compared to normal circulating B-cells [21].

All dogs also were required to undergo lymphadenectomy to confirm pathology [22], and provide material for the vaccine generation.

Ineligibility criteria included concurrent serious disorder (active systemic infection or second malignancies) that, in the opinion of the clinician, would compromise the ability to adhere to the protocol, previous therapy with any chemotherapeutic or immunotherapeutic agent, or glucocorticoids within the last 60 days.

The care of the dogs enrolled in the study was in accordance with institutional guidelines. All owners provided written informed consent.

2.3. Vaccine preparation

HSPPC was purified from LN specimens and prepared as a vaccine. The method of preparation is described in detail elsewhere [13]; Supplemental data]. Once prepared, the doses were kept frozen at −18 °C until use.

2.4. Treatment

Dogs in both treatment groups received the same 20-week combination chemotherapy, consisting of L-asparaginase (week 1), vincristine (week 2, 3, 4, 13), cyclophosphamide (week 2, 13), doxorubicin (week 7, 16), lomustine (week 10, 19), and prednisone (week 1 through 20), as previously described [13].

Dogs whose owners wished to pursue immunotherapy also received an intradermal injection of 0.5 ml vaccine on weeks 4–7, 12, 16, 20, and 24. The injection areas were shaved and aseptically prepared prior to vaccine administration. Vaccines were administered utilizing a 22 gauge needle that was 0.7 mm × 30 mm in length. For 30 min after the first injection, each dog was monitored for signs of skin irritation; vital signs were recorded before the injection and just before the dog left the clinic. All owners were asked to record and report adverse effects upon their visit for the next vaccine administration or to immediately report by telephone if serious events occurred.

Safety was assessed at each scheduled treatment session using the VCOG-CTCAE criteria [23]. Treatment was delayed for a maximum of 1 week or dose was decreased by 20% for safety changes. Concomitant medications, including antibiotics, antiemetic and antidiarrheal, were permitted to manage adverse events.

2.5. Response assessment and follow-up

Response was evaluated at each treatment session according to previously published criteria [24]. Responses were required to last for at least 28 days.

Two weeks after having completed chemotherapy and following immunotherapy, if administered, all dogs underwent restaging by repeating the initially altered examination. For MRD monitoring, FC on PB, BM and a LN aspirate, and/or PARR on PB, BM and LN obtained from a second lymphadenectomy was carried out [25]. Dogs were then rechecked through monthly physical examinations during the first year, and every other month thereafter. Dogs having initially visceral, PB and/or BM involvement also underwent imaging or FC testing every three months. Also, owners were asked to immediately seek for medical consultation in case of symptoms occurrence or LN enlargement.

Relapse was defined as clinical reappearance and cytological evidence of lymphoma in any anatomical site in dogs having experienced CR, whereas relapse for animals with PR was defined as progression.

Dogs that relapsed during or after the treatment protocol were offered standardized rescue chemotherapy.

2.6. Immunological monitoring

In vivo immune responses were documented in vaccinated dogs by performing DTH skin tests and by evaluating the local inflammatory response after the eighth vaccination (Supplemental data)

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[26]. An induration at the injection site lasting >48 h was considered a DTH response. Any induration or erythema at the injection site clearing within 48 h was considered to be a negative response.

2.7. Statistical analysis

Descriptive statistics were used in the analysis of dogs' characteristics, response rates and adverse events. When appropriate, data sets were tested for normality by use of the D'Agostino and Pearson omnibus normality test. Values were expressed as mean ± standard deviation in case of normal distribution, or as median with a range in case of non-normal distribution.

Demographic data (age, sex), histotype (MZL, FL, B-SLL) and documented negative prognostic factors (stage, substage, anemia [PCV < 35], thrombocytopenia [PLT < 150,000/μl], hypercalcemia [calcium > 11.2 mg/dl], increased LDH level [LDH > 270 u/l]), and weight were analyzed to detect any possible statistically significant difference between the 2 groups. Differences were evaluated with Student's *t* test/Mann–Whitney *U* test (continuous variables) and Fisher's exact test (categorical variables).

Survival analysis was performed to evaluate differences in TTP and LSS between vaccinated and unvaccinated dogs. TTP was measured as the interval between initiation of treatment and PD, whereas LSS was measured as the interval between initiation of treatment and lymphoma-related death (meaning all fatalities due to lymphoma, including euthanasia) [24].

All dogs were included in TTP and LSS analysis to fulfill intention-to-treat criteria. Dogs lost to follow-up and dogs that died due to lymphoma-unrelated causes or treatment were right-censored at the last date of known status or when they died from other causes, respectively.

Curves for TTP were generated according to the Kaplan–Meier product-limit method. Other variables were entered in survival analysis for potential prognostic significance, including sex, age (≤ or > median value), body weight (≤ or > median value), histotype (MZL, FL, B-SLL), stage, substage, anemia, thrombocytopenia, LDH, and toxicity. The log-rank test was used to compare survival distributions.

Statistical calculations were performed using SPSS Statistics v.19 (IBM, Somers, NY, USA). The variables with a *P*-value <0.1 were further evaluated for their independence by use of the Cox proportional hazard model. For each variable, the risk (hazard ratio) of progression or lymphoma-related death was estimated with corresponding 95% confidence intervals (CIs).

Significance was set at $P < 0.05$.

3. Results

Forty-five dogs were enrolled. Table 1 provides a summary of dogs' characteristics using known or potential outcome variables. Although dogs were not stratified based on prognostic risk, there was good balance between arms regarding possible outcome variables. Only histotype tended to be significantly different among groups ($P = 0.053$), with B-SLLs being more often diagnosed in Group 1, and FLs in Group 2.

Twenty dogs were treated with dose-intense chemotherapy (Group 1), and 25 received concurrent immunotherapy (Group 2).

Eleven (57.9%) dogs in Group 1 and 18 (72%) dogs in Group 2 completed the chemotherapy protocol. Reasons for not completing chemotherapy in Group 1 were attributable to PD ($n = 8$) or chemotherapy-related side effects ($n = 1$).

Vaccines were successfully prepared for all 25 dogs in Group 2, and a total of 187 doses were administered (median 8 doses/dog; range, 5–8). In this group, 4 dogs experienced PD and ceased treatment before all of their vaccination doses were administered. Other tumor-unrelated causes for not finishing the protocol included gastric dilation/volvolus ($n = 1$), car accident ($n = 1$), and poisoning with rodenticides ($n = 1$).

Table 1
Baseline characterization of dogs receiving chemotherapy (Group 1) or chemo-immunotherapy (Group 2) for known and potential covariates of outcome in canine lymphoma.

Variable	Group 1 ($n = 20$)	Group 2 ($n = 25$)	*P*-value
Age (years; mean)	8.4 ± 3.5	7.7 ± 2.9	0.423
Sex			0.920
Male ($n = 24$)	11	13	
Female ($n = 21$)	9	12	
Weight (kg; mean)	23.8 ± 14.7	22.4 ± 12.6	0.741
Histology			0.053
MZL ($n = 29$)	14	15	
FL ($n = 11$)	2	9	
B-SLL ($n = 5$)	4	1	
Stage			0.766
IV ($n = 10$)	4	6	
V ($n = 35$)	16	19	
Substage			0.823
a ($n = 33$)	15	18	
b ($n = 12$)	5	7	
PCV			0.495
Normal ($n = 43$)	20	23	
Decreased ($n = 2$)	0	2	
PLT			0.577
Normal ($n = 42$)	18	24	
Decreased ($n = 3$)	2	1	
Calcium			0.444
Normal ($n = 44$)	19	25	
Increased ($n = 1$)	1	0	
LDH			0.543
Normal ($n = 29$)	14	15	
Increased ($n = 16$)	6	10	
Follow-up (days, median)	626	795	0.135

3.1. Clinical outcome and prognostic factors

Of the unvaccinated dogs, 9 achieved CR, 7 achieved PR, and 4 experienced PD. Median time to obtain CR and PR was 12 days (range, 1–19 days) and 9 days (range, 2–9 days), respectively.

Of the vaccinated dogs, 22 achieved CR and 3 achieved PR. Median time to obtain CR and PR was 3 days (range, 1–21 days) and 2 days (range, 2–3 days), respectively.

The proportion of dogs obtaining CR and/or PR was significantly higher in Group 2 than in Group 1 ($P = 0.032$).

For all dogs, median TTP was 179 days (range, 1–533 days). TTP was significantly longer in Group 2 than in Group 1 ($P = 0.015$). Median TTP for dogs receiving chemo-immunotherapy was 209 days (range, 29–533 days) versus 85 days (range, 1–300 days) for dogs receiving chemotherapy (Table 2). Kaplan–Meier curves for TTP are shown in Fig. 1. On both univariate and multivariate analyses, the received treatment was the only variable significantly influencing TTP (Tables 2 and 3).

At relapse, 2 cases of B-SLL (one in either treatment group) underwent transformation to DLBCL, which was histologically confirmed, with an overall incidence of transformation of 4.4%, and of 40% within the group of B-SLL.

At data analysis closure, 3 of 20 (15%) dogs in Group 1 were still alive with a median follow-up of 240 days (range, 234–583 days). Fourteen dogs had died due to lymphoma, 2 had died because of chemotherapy-related side effects, whereas 1 dog was killed by another dog.

In Group 2, 5 of 25 (20%) dogs were still alive, with a median follow-up of 507 days (range, 341–816 days). Fifteen dogs had died due to lymphoma, whereas 5 dogs died for lymphoma-unrelated causes.

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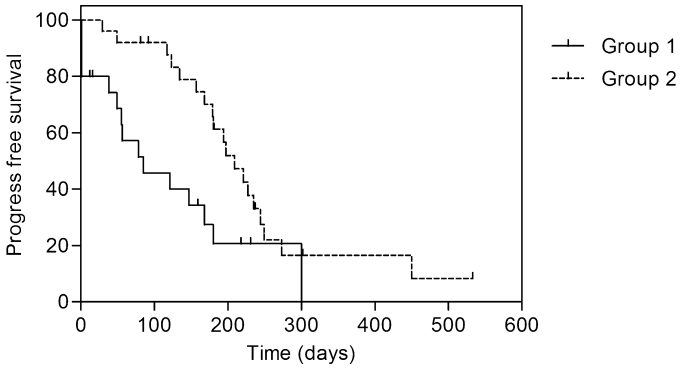

Fig. 1. Kaplan–Meier curves comparing unvaccinated Group 1 to vaccinated Group 2 for TTP.

Table 2
Median TTP for known and potential covariates of outcome in 45 dogs with indolent lymphoma.

Variable	All cases ($n=45$) Median TTP (days)	*P*-value
Age		0.577
≤8 years ($n=26$)	179	
>8 years ($n=19$)	180	
Sex		0.798
Male ($n=24$)	194	
Female ($n=21$)	157	
Weight		0.869
≤24.6 kg ($n=23$)	147	
>24.6 kg ($n=22$)	194	
Histology		0.088
MZL ($n=29$)	157	
FL ($n=11$)	235	
B-SLL ($n=5$)	117	
Stage		0.680
IV ($n=10$)	168	
V ($n=35$)	180	
Substage		0.254
a ($n=33$)	197	
b ($n=12$)	134	
PCV		0.972
Normal ($n=43$)	168	
Decreased ($n=2$)	180	
PLT		0.067
Normal ($n=42$)	180	
Decreased ($n=3$)	56	
LDH		0.355
Normal ($n=29$)	168	
Increased ($n=16$)	194	
Toxicity		0.234
No ($n=26$)	157	
Yes ($n=19$)	197	
BM toxicity		0.169
No ($n=34$)	168	
Yes ($n=11$)	197	
Molecular remission		0.320
No ($n=13$)	209	
Yes ($n=8$)	179	
Treatment		0.015*
Chemotherapy ($n=20$)	85	
Chemo-immunotherapy ($n=25$)	209	

* Significant.

Table 3
Multivariate analysis of potentially negative prognostic factors affecting TTP in 45 dogs with indolent lymphoma.

Variable	HR	95% CI	*P*-value
Chemotherapy only	2.26	1.10–4.66	0.026*
Thrombocytopenia	3.11	0.91–10.69	0.071
Nonfollicular lymphoma	2.12	0.84–5.37	0.113

* Significant.

Table 4
Median LSS for known and potential covariates of outcome in 45 dogs with indolent lymphoma.

Variable	All cases ($n=45$) Median LSS (days)	*P*-value
Age		0.123
≤8 years ($n=26$)	359	
>8 years ($n=19$)	248	
Sex		0.844
Male ($n=24$)	349	
Female ($n=21$)	330	
Weight		0.187
≤24.6 kg ($n=23$)	341	
>24.6 kg ($n=22$)	248	
Histology		0.680
MZL ($n=29$)	248	
FL ($n=11$)	330	
B-SLL ($n=5$)	347	
Stage		0.237
IV ($n=10$)	302	
V ($n=35$)	341	
Substage		<0.001*
a ($n=33$)	349	
b ($n=12$)	125	
PCV		0.866
Normal ($n=43$)	337	
Decreased ($n=2$)	330	
PLT		0.019*
Normal ($n=42$)	341	
Decreased ($n=3$)	127	
LDH		0.963
Normal ($n=29$)	337	
Increased ($n=16$)	349	
Toxicity		0.902
No ($n=26$)	341	
Yes ($n=19$)	296	
BM toxicity		0.175
No ($n=34$)	330	
Yes ($n=11$)	337	
Molecular remission		0.002*
Yes ($n=8$)	1042	
No ($n=13$)	337	
Treatment		0.173
Chemotherapy ($n=20$)	200	
Chemo-immunotherapy ($n=25$)	349	

* Significant.

Median overall LSS was 337 days (range, 5–1042 days). Unvaccinated dogs had a median LSS of 200 days (range, 5–1042 days), whereas vaccinated dogs had a median LSS of 349 days (range, 81–816 days). There was no difference in LSS between groups ($P=0.173$; Table 4).

Among the other evaluated variables, factors found to be significantly associated with LSS were substage and thrombocytopenia (Table 4). Overall, dogs without symptoms ($n=33$) had a significantly ($P<0.001$) longer LSS (349 days), compared with symptomatic dogs ($n=12$; 125 days). Similarly, non-thrombocytopenic dogs ($n=42$) had a significantly ($P=0.019$) longer LSS (341 days), compared with thrombocytopenic dogs ($n=3$; 127 days). On multivariate analysis, both parameters were significant in the model (Table 5).

Table 5
Multivariate analysis of potentially negative prognostic factors affecting LSS in 45 dogs with indolent lymphoma.

Variable	HR	95% CI	*P*-value
Substage b	6.61	2.48–17.60	<0.001*
Thrombocytopenia	6.99	1.84–26.47	0.004*

* Significant.

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3.2. Delayed-type hypersensitivity skin test

After the last injection of the vaccine, DTH skin tests were performed on all dogs that were still alive at that point. Eighteen (72%) dogs were evaluable: among them, 13 (72.2%) had a positive DTH response, and the remaining 5 (27.8%) cases had an undetectable response.

Interestingly, median TTP was 244 days for dogs with a positive DTH response versus 180 days for dogs with undetectable response ($P = 0.012$). Additionally, dogs with a positive response survived significantly longer than those without a detectable response (399 days versus 296 days, respectively; $P = 0.003$).

3.3. Minimal residual disease monitoring

Five dogs in Group 1 being in clinical remission at the end of chemotherapy underwent MRD monitoring with FC ($n = 2$) or PARR ($n = 3$). MRD was detected in 2 dogs by PARR and in 1 dog by FC, whereas molecular remission was achieved in the 2 remaining dogs, according to FC ($n = 1$) and PARR ($n = 1$).

All dogs with MRD relapsed during the study, and 2 of them were dead due to their lymphoma at study analysis closure. One of the 2 dogs achieving molecular remission relapsed during the study, and the other was in durable first CR at analysis closure.

Fifteen dogs in Group 2 being in clinical remission at the end of chemotherapy underwent MRD monitoring with FC ($n = 4$) or PARR ($n = 11$). MRD was detected in 9 dogs by PARR and in 2 dogs by FC. Six dogs with MRD relapsed during the study, and 6 of these were dead due to their lymphoma.

Four dogs achieved molecular remission. These dogs relapsed during the study; two of them were alive at the end of the study.

Overall, dogs achieving molecular remission had a significantly longer LSS than those, which failed to achieve it ($P = 0.002$).

3.4. Toxicity

Safety was assessed in all dogs. The type, frequency, and severity of treatment-related adverse events were comparable between the 2 treatment arms. In agreement with a previous study [13], chemo-immunotherapy was well tolerated. In particular, vaccination was well tolerated, with no adverse effects observed. No difference in dose-limiting neutropenia, thrombocytopenia, and gastrointestinal or hepatic toxicity was seen in dogs treated with dose-intense chemotherapy or chemo-immunotherapy (Tables 2 and 4).

4. Discussion

Nowadays, there is no consensus about the best treatment for dogs with indolent lymphoma in differing situations, highlighting a lack of knowledge and of randomized studies.

Typically, the management of indolent lymphomas is mainly determined by tumor burden and substage. In frontline treatment, a watchful waiting policy remains a good option if the dog has no risk criteria or symptoms. In dogs having an advanced stage disease, chemotherapy is the best option, although it has not been established which strategy (dose-intense versus metronomic). It also remains to be determined whether after front-line treatment, maintenance plays a role instead of observation only [2]. It appears obvious that studies aimed at carefully allocating treatment according to disease presentation are warranted.

Indeed, eligibility criteria for clinical trial enrollment are extremely important if results of studies performed internationally are to be compared. A literature search identified three studies focusing on canine indolent nodal lymphomas [2,6,7]. Unfortunately, enrollment criteria, staging work-up, treatment and response assessment varied among these studies, thereby impeding comparisons. It may be possible that the fairly good prognosis attributed to indolent B-cell lymphomas by one study [6] only reflects a less advanced stage, thereby challenging the belief that dogs with indolent lymphoma are uniformly long-term survivors.

The present series treated and followed-up in two institutions having adopted the same staging and treatment protocol provided an opportunity to study a new therapeutic approach. All dogs included here had advanced disease at presentation: 10 dogs had visceral involvement (stage IV), and 35 dogs had PB and/or BM involvement (stage V). Compared to previously published results [5–7], the outcome of the present series appears disappointing, despite the use of aggressive therapy, highlighting the importance of staging in anticipating prognosis. Based on our results, even after aggressive therapy, there is no consistently observed plateau in the survival curves, suggesting that dogs with advanced stage disease are incurable with current approaches.

However, chemo-immunotherapy enhanced antitumor activity by significantly increasing TTP compared with the duration of TTP achieved with traditional chemotherapy (209 versus 85 days, respectively). Similarly, vaccinated dogs had a significantly higher response rate (CR or PR) than unvaccinated dogs (100% versus 80%, respectively). These results suggest that at least some dogs may benefit from immunotherapy, thereby limiting tumor escape after chemotherapy that typically gives rise to relapse. This trial also confirmed that chemo-immunotherapy is safe with no unacceptable toxicities.

At data analysis closure, 15% of dogs in Group 1 and 20% of dogs in Group 2 were still alive. Although LSS was not statistically different among groups, a longer follow-up is warranted to ultimately assess whether the introduction of immunotherapy in first-line regimens also results in improved LSS.

Notably, the lack of statistical significance in LSS between vaccinated and unvaccinated dogs may be due to factors other than vaccination efficacy, such as individual differences in eliciting effective immune responses [27].

Indeed, an interesting correlation between clinical and immune responses was highlighted here, showing a significantly increased TTP and LSS in dogs developing an immune response compared with dogs that did not mount such a response. This observation suggests that the dogs' immune milieu at the time of vaccination is central in determining the extent of an effective immune response. Thus, efforts should be made to increase immunogenicity and to identify suitable predictive biomarkers [27].

The statistical data regarding poor prognostic factors confirmed the relevance for LSS of substage b and thrombocytopenia. In the literature, clinical substage b and thrombocytopenia at diagnosis have been recognized in dogs with lymphoma as important prognostic factors for remission duration and survival [28–31].

Last, the achievement of molecular remission following conventional chemotherapy correlated with a significantly increased LSS, as it did following chemo-immunotherapy. Therefore, although difficult to achieve, molecular remission is attainable and is a relevant endpoint that correlated with a longer LSS.

4.1. This study poses the basis for further research

The majority of studies of active immunotherapy targeting canine B-cell lymphomas have been designed to verify the role of vaccination in avoiding recurrences during or after chemotherapy [32–34]. The rationale of this approach relies on the fact that the antitumor immune response promoted by the vaccine could be clinically effective only in presence of a limited tumor load, when the dog is close to a MRD status.

Based on our previous experience with canine DLBCL [13], immunotherapy was initiated quite early in the treatment protocol,

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regardless of the response to chemotherapy. Although most dogs were already in clinical CR, it may be possible that they still had MRD when immunotherapy was started. In agreement with human trials [35,36], immunotherapy administered to dogs in CR after chemotherapy may result in clearance of residual tumor cells and subsequent enhanced TTP and LSS. Conversely, the persistence of significant numbers of malignant cells could make the immune environment unfavorable for the development of an adequate immune response. The experience in the human field suggests the possibility to modify the vaccination schedule in canine trials to enhance vaccine response. The completion of induction chemotherapy might minimize early relapse and facilitate vaccination in most dogs. In this scenario, immunotherapy would be considered for a consolidative strategy aimed at providing effective therapy with no adjunctive toxicity, thereby delaying relapse and prolonging the time to next treatment.

Another issue that merits further investigation is how many vaccine doses should be administered to optimize treatment outcome. This study was not designed to address the possible role of long-term vaccination. Periodic booster vaccinations may be required to maintain the immune response, which subsequently keeps the tumor burden below a clinically detectable level.

Finally, disease transformation into a more aggressive histological type, typically represented by DLBCL, is a common terminal event in people [37,38]. The relationship between indolent lymphoma and DLBCL is incompletely understood, and there remains a paucity of data regarding the incidence and management of high-grade transformation [39]. Here, two dogs with B-SLL underwent DLBCL transformation, thereby underlying the importance of accurate initial diagnosis, long-term follow-up, and re-biopsy at relapse.

In conclusion, we demonstrated the efficacy of chemo-immunotherapy in prolonging TTP in dogs with advanced indolent B-cell lymphoma. Questions of whether the response elicited by immunotherapy is dose-dependent, whether the vaccine should be administered after induction chemotherapy with dogs in CR, whether any responses will be long-lasting, whether dogs benefit from periodic booster vaccination, and at what intervals remain unanswered. Undoubtedly, HSPs acting as peptide chaperones continue to be an important area of development in veterinary oncology.

Author contributions

LM, LA, PF and NR conceived the project. SS performed the statistical analysis for this evaluation, prepared the tables and figure. LM, DS, SC, FR, PL, SP and LA were responsible for recruitment and acquisition of data. LM wrote the paper.

All authors had full access to all of the data, and can take responsibility for the integrity of the data and the accuracy of the data analysis. All authors approved the final version to be published and are in agreement with the analysis, results and interpretation of the findings.

Conflict of interest statement

PD and NR are employees of Urodelia. This does not alter our adherence to all the policies on sharing data and materials. The remaining authors declare that no other competing interests exist.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2015.08.017.

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