

# **Contribution to the evaluation of clinical prognostic factors in canine lymphoma**

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## Resumo Alargado

O linfoma é o tumor hematopoiético mais frequente no cão com uma incidência estimada entre 13 a 114 casos por 100.000 cães em risco. A poliquimioterapia, através da utilização de um protocolo composto pelos fármacos Ciclofosfamida, Hydroxidaurorrubicina (Doxorrubicina), Oncovin® (Vincristina) e Prednisolona, designado pelo acrónimo de “CHOP”, permite obter uma taxa de remissão e tempo médio de sobrevida global de cerca de 94% e 12 meses, respectivamente.

O linfoma difuso de células B grandes (LDCBG) pode constituir até cerca de 50% dos casos totais de linfoma non-hodgkin (LNH) na espécie canina e é reconhecido como o modelo natural para o mesmo tipo de neoplasia no Homem.

A aplicação do esquema de classificação da Organização Mundial da Saúde nas entidades de linfoma canino (LC) é recente na medicina veterinária. A maioria dos estudos clínicos realizados no LC para avaliação da resposta à terapêutica, assim como a determinação de factores de prognóstico realizados nas últimas três décadas, sofrem de limitações significativas. Os resultados desses estudos, foram obtidos através da inclusão de diversos subtipos e graus histológicos de linfoma, assim como, da utilização de metodologias de diagnóstico, estadiamento e terapêutica variáveis. Estas limitações comprometem, ainda hoje, a avaliação da eficácia do tratamento e colocam em questão a fiabilidade de diversos factores de prognóstico descritos para o LC.

Na medicina humana e veterinária existem estudos que demonstram que a localização geográfica, a demografia, os factores sócio-económicos dos doentes e as diferenças na distribuição de subtipos de linfoma podem interferir na resposta à terapêutica e na determinação do prognóstico.

Em Portugal, existe um número muito reduzido de estudos sobre o LNH no cão. Que seja do conhecimento do autor, não existem trabalhos avaliando os aspectos clínicos, nomeadamente, no que diz respeito à resposta à terapia ou prognóstico.

A infecção por agentes infecciosos transmitidos por vectores (AITV), nomeadamente dos géneros Ehrlichia e Leishmania, aparece pontualmente na literatura associada a tumores hematopoiéticos nos cães. Estes agentes infecciosos promovem uma resposta inflamatória crónica do hospedeiro, que causa uma importante desregulação imunológica, contribuindo para o processo de carcinogénese, nomeadamente, para a linfomagénese ou progressão tumoral.

As manifestações clínicas das infecções AITV nos cães estão associadas a um amplo espectro de anomalias clínicas e laboratoriais. A presença de adenomegalia, hepato-

esplenomegalia, síndromes imunomediados (uveíte, glomerulonefrite, poliartrite, etc.), anemia, alterações no número e tipo de leucócitos circulantes ou trombocitopénia estão entre as mais frequentes.

Num cão que sofra de linfoma e que esteja, também, infectado por um ou mais destes agentes infecciosos, a interpretação adequada dos sinais clínicos e das alterações dos valores laboratoriais torna-se complexa, impedindo uma correcta diferenciação do que é devido à infecção, ao linfoma ou a ambos. Qualquer inferência de uma possível ausência de resposta à terapêutica oncológica instituída, assim como a determinação da presença de indicadores prognósticos específicos do linfoma torna-se difícil ou, mesmo, impossível.

Tal como em medicina humana, na medicina veterinária a obtenção de informação diagnóstica e/ou prognóstica num conceito “*bench-to-bedside*” é de extrema importância. A confirmação serológica ou molecular de infecção por AITV é, actualmente, rápida e está amplamente disponível. A presença de laboratórios comerciais de patologia clínica veterinária ou a disponibilização nos hospitais e clínicas veterinárias de equipamentos de hematologia com tecnologia avançada permitem, ainda, obter rapidamente um hemograma com alto nível de fiabilidade nas contagens diferenciais das populações celulares.

Na oncologia clínica humana e, posteriormente, na veterinária os valores obtidos no momento do diagnóstico, através da contagem diferencial de alguns componentes celulares do sangue periférico (neutrófilos, linfócitos, monócitos, e plaquetas), bem como os rácios neutrófilos-linfócitos (NLR), linfócitos-monócitos (LMR), demonstraram ter valor prognóstico ao reflectir as interfaces inflamatórias intra-neoplásica e doente-tumor no LDCBG.

Tendo em consideração a literatura publicada em medicina humana sobre o valor prognóstico da composição celular e dos rácios NLR e LMR, obtidos a partir do sangue periférico no momento do diagnóstico do LDCBG, e a escassa ou contraditória informação nesse domínio na área veterinária, foram objetivos do presente trabalho: 1) investigar a prevalência de infecção por quatro agentes transmitidos por vectores (*Leishmania infantum*, *Ehrlichia canis*, *Anaplasma phagocytophilum* e *Bartonella henselae*), o seu potencial papel na linfomagénese e a sua eventual associação com o subtipo do tumor e com as alterações hematológicas presentes em cães diagnosticados com linfoma e 2) determinar o valor prognóstico dos parâmetros sexo, estado fértil, estágio clínico do linfoma, presença de anemia, presença de neutrofilia, presença de trombocitopénia, NLR, LMR e dois rácios adicionais, nunca descritos- plaquetas-linfócitos e plaquetas-neutrófilos- em cães com LDCBG em estágio III/IV tratados com um protocolo CHOP de 19 semanas.

Relativamente ao primeiro estudo, foi avaliada pela primeira vez (em Portugal e na Europa) a associação entre a infecção por quatro agentes transmitidos por vectores (*Leishmania infantum*, *Ehrlichia canis*, *Anaplasma phagocytophilum* e *Bartonella henselae*) em cães com linfoma.

A presença de anticorpos (IgG) assim como a detecção molecular de *Bartonella henselae*, *Ehrlichia canis* e *Anaplasma phagocytophilum* foram negativas em todas as amostras de cães com linfoma estudadas. Para *Leishmania infantum* a taxa de seropositividade e de detecção molecular foi de, respectivamente, 8,2% e 3,3%. Estes valores não se mostraram significativamente diferentes quando comparados com os controlos históricos de cães com o mesmo estilo de vida e da mesma região geográfica: 7,9% ( $p = 0,201$ ) e 1,2%, ( $p = 0,166$ ), respectivamente. Dos 8,2% de cães seropositivos para *Leishmania infantum*, 60% tinham diagnóstico de LDCBG. Os 40% restantes desenvolveram formas de linfoma indolentes (Manto e de Pequenas células).

Relativamente às alterações hematológicas nos cães seropositivos para *Leishmania infantum* (n=5), apenas um cão, portador de linfoma de pequenas células B, não apresentou qualquer alteração hematológica. A presença de anemia leve foi observada em dois cães, um deles com trombocitopenia concomitante. Verificou-se, ainda, a presença de monocitopenia e linfopenia moderadas em dois cães diferentes. Todas as alterações do sangue periférico observadas nestes cães foram leves e não associadas ao tipo de linfoma, título serológico ou positividade molecular para *Leishmania infantum*.

No presente estudo não foi possível diferenciar se essas alterações hematológicas estão relacionadas com o linfoma e/ou com a infecção por *Leishmania infantum*. Provavelmente, as alterações dos hemogramas dos cães com linfoma e com leishmaniose devem-se à ação de ambas as doenças e resposta inflamatória associada, quer na produção, quer na cinética dos elementos celulares sanguíneos, ao nível da medula óssea e do sangue periférico.

Os cães com leishmaniose, quando comparados com a população geral do estudo, apresentaram um Tempo para a Progressão (TPP) e uma Sobrevivência Específica para o Linfoma (SEL) mais curtos, 90 vs. 120 e 184 vs. 206 dias, respectivamente. Devido ao número muito pequeno de cães positivos, não foi possível obter qualquer conclusão significativa sobre a associação dos tipos específicos de linfoma e o impacto nas anomalias das células do sangue periférico, assim como a sua possível implicação na linfomagenese.

Relativamente ao segundo objectivo, foram avaliados o valor prognóstico dos parâmetros sexo, estado fértil, estágio clínico, presença de anemia, presença de neutrofilia, presença de trombocitopenia, NLR, LMR e os rácios plaquetas-linfócitos (PLR) e plaquetas-neutrófilos (PNL) relativamente ao TPP e SEL. Foram, ainda, calculados para dois marcos temporais específicos (6 e 12 meses) a Taxa do Tempo de Progressão (TTPP) e Taxa de

Sobrevivência Específica do Linfoma (TSEL). Estes segundos cálculos permitem uma compreensão mais clara da evolução do TPP e do SEL.

O PNR revelou-se um marcador prognóstico independente ( $p \leq 0,001$ ) para a TTPP aos 180 e 365 dias. Os cães com PNR acima de 0,032 apresentaram maior probabilidade de progressão do linfoma antes de 180 dias (sensibilidade 46,5%, especificidade 87,5%,  $p = 0,004$ ). O NLR mostrou uma significância prognóstica para a TSEL aos 180 ( $p = 0,006$ ) e 365 dias ( $p = 0,009$ ). Um valor de NLR ao diagnóstico abaixo de 7,45 mostrou estar associado positivamente a uma maior probabilidade de sobrevida aos 180 dias (sensibilidade de 52%, especificidade de 85,3%,  $p = 0,025$ ).

Relativamente à análise de outros factores de prognóstico anteriormente validados, no presente estudo a presença do sub-estádio b, demonstrou contribuir para a progressão precoce do linfoma e diminuição da sobrevida aos 180 dias ( $p = 0,031$ ). Nos cães com anemia, designadamente com concentrações de hemoglobina inferiores a 11 g/dL, a TSEL aos 365 dias foi significativamente inferior aos cães com valores de concentração de hemoglobina normais ( $p = 0,028$ ).

No presente estudo, os cães com linfoma na região metropolitana de Lisboa apresentaram, para os agentes infecciosos estudados, a mesma seroprevalência e taxa de detecção molecular que os controlos históricos da mesma zona geográfica e com o mesmo estilo de vida. Este primeiro estudo foi de extrema importância para permitir compreender a magnitude da infecção pelos quatro AITV estudados nos cães com linfoma e o eventual impacto no subtipo morfológico, alterações hematológicas e possível contribuição para a linfomagénese.

Os presentes resultados não revalidaram factores de prognóstico anteriormente descritos para o linfoma multicêntrico no cão, como o género, o estado fértil e, no caso particular do LDCBG, o LMR, a neutrofilia e trombocitopenia ao diagnóstico. O rácio neutrófilo-linfócito demonstrou valor prognóstico na sobrevida total, como anteriormente descrito.

Este é o primeiro estudo a realizar a avaliação dos factores PLR e PNR em cães com LDCBG e a demonstrar que o PNR ao diagnóstico, tem um valor predictivo da progressão precoce do linfoma.

Uma vez que a composição celular do sangue periférico pode ser afetada por várias causas não oncológicas, inflamatórias ou infecciosas, será importante continuar o desenvolvimento de estudos prospectivos multicêntricos com critérios de inclusão homogéneos e com um número significativo de doentes, de forma a determinar a verdadeira associação entre os AITV e a dinâmica da composição celular periférica, nos cães com LDCBG tratados com CHOP.

O presente estudo adiciona dados inovadores e importantes para melhor compreender a epidemiologia do linfoma canino e, particularmente, o espectro prognóstico do LDCBG.

Os indicadores de prognóstico aqui descritos estão amplamente acessíveis, são de rápida obtenção e baixo custo, permitindo uma abordagem “*bench-to-bedside*” na estratificação de risco dos pacientes e, conseqüentemente, o desenvolvimento de abordagens terapêuticas personalizadas.

Uma janela para a suposta contribuição de *Leishmania infantum* para a linfomagénese permanece aberta e constitui um ávido assunto de pesquisa que merece ser continuado.

**Palavras-chave :**

Linfoma no cão

Linfoma difuso de células B grandes

Factores de prognóstico

Hematologia

Rácios hematológicos

Doenças transmitidas por vectores



# Abstract

Non-Hodgkin Lymphoma (NHL) is the most common hematopoietic cancer in dogs, from which up to 50% of the cases are diffuse large B-cell lymphomas (DLBCL). The etiology, like in humans, is believed to be multifactorial. To the date, the best response rate and best survival times are offered by a combination therapy including cyclophosphamide, vincristine, doxorubicin and prednisolone, known by the acronym of CHOP.

Infectious agents, namely vector-borne agents (VBA), can induce chronic B cell stimulation and immune deregulation permitting lymphomagenesis. Also, there are several reports in literature associating NHL with VBA, namely from the genus *Borrelia* and *Leishmania* mimicking or co-existing with hematopoietic malignancies either in humans or dogs. Vector-borne agents can induce haematological and clinical changes in hosts that, when existing in cancer patients, can either mislead the interpretation of clinical signs or interfere with recognized prognostic markers, namely blood cell populations.

Prognosis, after quality of life, is determinant in veterinary oncology to further proceed with a treatment. Short survival times and therapy response rates determine the option for a non standard-of-care treatment or, lately, animal euthanasia without treatment. Consequently, easy to perform, “*bench-to-bedside*” widely available and cost-effective prognostic markers are fundamental to obtain client financial compliance and support treatment planning and disease outcome.

Obtaining serological results on vector-borne agents or haematological information through a total blood cell count and biochemical parameters are widely available and cost effective either by an “in-house” laboratory or through commercial laboratories.

Having in mind the existing published literature on human medicine regarding haematological parameters as prognostic indicators in lymphoma and the scarce information on the veterinary field, we aimed to: 1) investigate the prevalence of infection by four vector-borne agents (*Leishmania infantum*, *Ehrlichia canis*, *Anaplasma phagocytophilum* and *Bartonella henselae*), its potential role in lymphomagenesis and its possible association with the tumour subtype and with the haematological alterations present in dogs with lymphoma and, 2) determine the prognostic value of dogs’ sex, neutered status, clinical stage, presence of anaemia, presence of neutrophilia, presence of thrombocytopenia and the ratios lymphocyte-to-monocyte (LMR), neutrophil-to-lymphocyte (NLR), platelet-to-lymphocyte (PLR) and platelet-to-neutrophil (PNR) in canine DLBCL that were naïve for treatment, fully staged and received chemotherapy with a 19 week-CHOP protocol.

All dogs tested negative for *B. henselae*, *A. phagocytophilum* and *E. canis* by both serology and molecular detection. Regarding *L. infantum*, 8,2% of the dogs had a positive serologic result. *Leishmania infantum* DNA was detected in two samples of diffuse large B-cell lymphoma (DLBCL). These results show an increased, but not significant, seropositivity (8,2%,  $p=0,201$ ) and molecular detection (3,3%,  $p=0,166$ ), for *L. infantum* in dogs with lymphoma, when compared to matched historic controls in the same geographical area.

In the second study, PNR showed to be an independent prognostic marker ( $p\leq 0,001$ ) for TTPR at 180 and 365 days. Dogs with a PNR above 0,032 were more likely to progress before 180 days (sensitivity 46,5%, specificity 87,5%,  $p=0,004$ ). On univariate analysis, NLR showed a prognostic significance for LSSR at 180 ( $p=0,006$ ) and 365 days ( $p=0,009$ ). A baseline NLR value below 7,45 was positively associated with survival at 180 days (sensitivity of 52%, specificity of 85.3%,  $p=0.025$ ). The presence of substage *b*, was associated with early lymphoma progression and decreased survival at 180 days ( $p=0.031$ ). Anaemia significantly reduced LSSR at 365 days ( $p=0,028$ ).

Although it was not possible to identify, in the first study, any significant association between canine lymphoma and the studied VBA, it was of extreme importance for the discussion of the second study on the possible effects on peripheral blood cell dynamics caused by the CBVD studied. Further studies, following dogs through their CVBD disease evolution, are worthwhile and may help clarify a possible role of these agents in lymphomagenesis.

This is the first study evaluating PLR and PNR in canine DLBCL and demonstrates that PNR could be a predictor of early lymphoma progression. Since peripheral blood cell composition can be affected by several non-oncological causes, the development of larger multicenter studies with homogeneous inclusion criteria could help to better determine the true predictive values of blood cell ratios in dogs suffering from DLBCL treated with CHOP chemotherapy.

## **Keywords**

Canine lymphoma

Diffuse large B cell lymphoma

Prognostic factors

Haematology

Blood cell ratios

Vector-borne diseases



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# 1. General Introduction

Cancer exists probably since eukaryotic life started, around 1,6 million years ago,<sup>1</sup> but the first and oldest known scientific report reporting cancer in ancient ages is the surgical approach to breast cancer in the Edwin Smith papyrus dated from 1600BC.<sup>2</sup>

Dog has been always described as human's best friend. From sharing caves in the upper Palaeolithic period,<sup>3</sup> to herds, fights, farms, houses and, nowadays, the sofa and bed. The social, functional, protective and emotive relationship between Humans and (their) dogs is indubitable.<sup>4</sup> One may also think that dogs and humans may have started to suffer and die from cancer in the same evolutionary moment, and this was another common living experience of each one's life that still didn't end today.

Cancer is the second leading cause of Human death worldwide, and was responsible for 9.6 million human deaths in 2018. Globally, about 1 in every 6 human deaths is due to cancer.<sup>5</sup>

Human non-Hodgkin Lymphoma (hNHL) ranks eleven in the total number of new cancer cases with 509.590 new cases in 2018 and a total death of 248.724 of the affected patients (48,8%).<sup>6</sup>

Human Diffuse large B-cell lymphoma (hDLBCL) is the most common lymphoid malignancy in adults, accounting for 31% of all non-Hodgkin-lymphoma (NHL) cases in the Western world. It's clinical presentation, clinical behaviour and prognosis are variable and despite its aggressiveness it is potentially curable.<sup>7</sup>

The International Prognostic Index (IPI), age adjusted IPI (aaIPI) and National Comprehensive cancer Network-IPI (NCCN-IPI) are the benchmarks for prognosis determination.<sup>8</sup>

Immunochemotherapy with multidrug combination protocol, designated by CHOP (Cyclophosphamide-Hydroxydaunorubicin-Vincristine and Prednisolone) potentiated with anti-CD20 monoclonal antibody is the state-of-art treatment for hDLBCL patients.<sup>7</sup> Rituximab, an human/murine chimeric anti-CD20 approved 20 years ago, for treatment of CD20+ malignancies, when associated to CHOP chemotherapy has significantly improved overall survival.<sup>9,10</sup>

Canine Lymphoma (cL) resembles hNHL and its true incidence is unknown, varying with reports, but is estimated to be between 13 to 114 cases in 100.000 dogs at risk.<sup>11</sup> Multiagent chemotherapy with CHOP based protocols is the standard of care to treat cL with median remission rates and survival times around 94% and 9 months, respectively.<sup>12</sup> Despite the good response to therapy the cure rates are rare.<sup>13</sup> Canine Diffuse Large B-cell lymphoma (cDLBCL) accounts for 40%-50% of cases of canine NHL.<sup>14,15</sup> The application of World Health

Organization (WHO) lymphoma classification scheme and its use to identify lymphoma subtypes is recent in veterinary medicine<sup>15</sup> and the great majority of the studies assessing treatment responses and prognosis in the last three decades, have evidenced several limitations: mixture of lymphoma subtypes or grades, variable diagnostic methodology, use of non consistent staging procedures and assessing different clinical end-points. These limitations do not allow the real evaluation of treatment efficacy and accuracy in determining factors of prognosis.<sup>16</sup> Nevertheless, there are features that became constant and validated all throughout the time: T-cell lymphomas, (apart T-Zone Lymphoma), WHO clinical stage and substage b disease, have been always associated with worse response to therapy.<sup>17,18</sup>

Richard Dorn, in 1972 wrote about "Dogs, Cats and Man": "Where similarities exist, knowledge about a disease condition in one species may be applicable to another"<sup>19</sup> and if in 1972 this was already acknowledged, the development of new technologies that permit to assess the genetics, development, progression, immunology and therapy of cancer in both animals and humans, have increased in importance and strengthened the field of comparative oncology.<sup>20</sup>

This acquired knowledge on similarities in molecular aspects, signalling, incidence and pathologic features between canine and human NHL, strongly supports the utilization of canine lymphoma as a comparative, the large animal model for human NHL.<sup>21,22</sup>

DLBCL being the most common form of B-cell neoplasm in man and dog, it is crucial from a clinical point of view to carry on developing strong basic and clinical research to consolidate our knowledge on this disease.

Since the 17<sup>th</sup> century that the relationship between cancer and inflammation is recognized.<sup>23</sup> Inflammation and infections are documented as co-factors of immune deregulation,<sup>19</sup> a known crucial determinant in lymphomagenesis. Aggressive lymphoma subtypes, such as diffuse large B-cell lymphomas, are increasingly linked with features of systemic immune stimulation including autoimmune/inflammatory diseases.<sup>24</sup> Also, inflammation is an intrinsic component of tumour microenvironment and an enabling characteristic of cancer, contributing to tumour proliferation, cell migration, tissue invasion, and metastatic disease.<sup>25</sup> Evaluation of the subtype and number of peripheral blood cells at diagnosis and during treatment can reflect inflammatory conditions within the neoplasm and from the host immune system-tumour interaction. Parameters such as total white blood cell count and specific cellular classes as neutrophils, lymphocytes, monocytes, erythrocytes and platelets counts, as well as the ratios neutrophil-to-lymphocyte (NLR), and lymphocyte-to-monocyte (LMR), can be indicators of systemic inflammatory response and have been widely proposed as prognostic factors for many malignancies, including human<sup>26,27</sup> and canine lymphoma.<sup>28</sup>

Infectious agents, namely vector-borne agents (VBA), can induce in the host an immune response based inflammation that can lead to immune deregulation permitting lymphomagenesis.<sup>29,30</sup>

There are several reports in literature associating NHL with VBA, namely from the genus *Borrelia* and *Leishmania* mimicking or co-existing with hematopoietic malignancies either in humans<sup>31-34</sup> or dogs.<sup>35-38</sup> Vector-borne agents can induce haematological and clinical changes in hosts, that, when existing in cancer patients, can either mislead the interpretation of clinical data and symptoms or, interfere with recognized prognostic markers.<sup>39-42</sup>

Cancer diagnosis and treatment is associated with high costs in human and veterinary medicine. Access to adequate diagnostic methodology, staging procedures and treatments for pets, can be strongly conditioned by the financial situation of the animal guardian. Prognosis is a strong if not, the major, element of the equation when it is needed to decide the treatment plan. The presence of a non-favourable prognosis may determine the option for a non standard-of-care treatment or, lately, animal euthanasia without treatment, if solid prognostic information cannot substantiate the investment vs. the outcome.

Easy to perform, widely available and cost effective prognostic markers are needed to support treatment planning and associated outcome. Obtaining serological results on vector-borne agents or, haematological information through a total blood cell count and biochemical parameters are widely available and cost effective either by an "in-house" laboratory or through commercial laboratories.

Having in mind the existing published literature on human medicine regarding haematological parameters as prognostic indicators in lymphoma and the scarce information on the veterinary field, we sought to investigate: 1) a putative association between vector-borne agents and cL; 2) which of the haematological parameters described for humans may have a role in prognostication on cDLBCL patients that were naïve for treatment, fully staged and received the same therapy with a 19 week-CHOP based protocol.

## **1.2. Lymphoma, Lymphosarcoma or Malignant lymphoma**

### *1.2.1 Disease definition*

The first description of lymphoma in a dog was done by Siedamgrotzky in 1871.<sup>43</sup> The terms malignant lymphoma, lymphosarcoma or lymphoma are commonly used to describe the solid neoplastic proliferation of lymphoreticular cells. Canine Lymphoma is the term most commonly applied among the veterinary community and generally accepted in scientific literature.<sup>13,44,11</sup>

Lymphoma arises from normal lymphocytes, that are typically round cells produced during embryogenesis and foetal life in the yolk sac, foetal liver and bone marrow. After birth, lymphoid progenitors are situated in the primary lymphoid organs, such as bone marrow and Peyer's patches for B cells and thymus for T cells. Primary lymphoid organs produce and regulate the maturation of lymphocytes. Secondary lymphoid organs arise just before birth and consist of lymph nodes, tonsils and some lymphoid tissue scattered in the body, such as on the urinary, respiratory and digestive tracts. The mature lymphocytes, in the presence of Antigen Presenting dendritic Cells (APC's), are submitted to positive and negative selection to ensure that only cells that carry functional B Cell Receptor (BCR) and T Cell Receptors (TCR) are viable.<sup>45</sup> As important players in host defence, lymphoid cells can freely circulate in the body, being 50% of total peripheral blood leucocyte count.<sup>46</sup> It is estimated that lymph nodes home 40% of the total body lymphocyte population, followed by the spleen, with 13%, and the bone marrow, accounting only with 10%. Age plays a major role in determining primary lymphoid organs senescence (thymus involves at sexual maturity and bone marrow is replaced largely by fatty tissue) and regulating immune cells subsets. Age plays also an important control in the selection of phenotypes of the circulating lymphocyte subpopulation, differing considerably in neonatal and adult dogs. Newborns have high levels of circulating B cells that decrease with ageing. The circulating blood of a new born puppy has a lower proportion of T cells, with a very low rate of CD8+ T cells, and a high amount of B cells. The percentage of blood CD4+ T cells remains relatively stable through the life stages and CD8+ T cells quantity increases with age.<sup>46</sup> When lymphoma develops lymph nodes are the most common affected organs, but no place in the body is immune to harbour a lymphoid tumor.<sup>47</sup>

### 1.2.2 Epidemiology

Epidemiological studies in veterinary medicine are strongly limited by the absence of animal population census and registries and aggravated by the absence of non-obligatory declaration diseases such as cancer. The existing animal cancer registries, based upon estimated populations such as veterinary teaching hospitals,<sup>48</sup> insured animals<sup>49</sup> and geographical areas,<sup>50,51,52,53</sup> are not completely representative of the tumour cases. One should carefully interpret the incidence rates of different animal tumour registries, because the populations that serve for reference are obtained differently, what is strongly opposite to human studies where population is obtained through census. Despite descriptive important information, reported studies shown variations in tumour occurrence among different geographic areas, probably due to breed variations<sup>54</sup> or individual characteristics such sex<sup>55</sup> and different environmental factors, such as exposure to pollutants, ultraviolet radiation or access to veterinary care.<sup>53</sup>

The reported incidence rates of canine lymphoma can vary from 13 to 114 per 100.000 dogs at risk.<sup>52,13</sup> Canine Non-Hodgkin lymphoma (cNHL) accounts for 7% up to 24% of all canine malignant tumours, and constitutes 83% of all hematopoietic cancers.<sup>11</sup> The age-adjusted overall incidence of cNHL is 1.5 per 100 000 for dogs younger than 1 year and 84 per 100 000 for dogs older than 10 years.<sup>56</sup> One common finding is that incidence rates increase greatly in older dogs, aged  $\geq 8$  years old<sup>57</sup> with apparent exception in T cell lymphoma, where dogs aged less than 3 years seem to have an increased incidence.<sup>58</sup>

Breed also plays an important role in the epidemiology of cL, since the prevalence of the disease and associated risk, might be extremely influenced by heritable traits<sup>58</sup> and pure-breed dogs limited genetic variability enables genetic linking.<sup>59</sup> More specifically, Basset Hounds, St Bernard, Scottish Terrier, Airedale Terrier, Bulldog, Labrador Retriever, Bouvier des Flandres, Golden Retrievers, Bull Mastiffs, Doberman, Burmese Mountain Dog, Australian Cattle dog and Rottweiler are among the most affected breeds.<sup>54,60,61</sup> Non-Hodgkin lymphoma phenotype seems to be associated with some breeds, with Spitz and Shih tzu having increased risk of T cell tumours or Mastiff, Herding dogs and recent European breeds being prone to B-cell phenotype.<sup>60</sup> In the specific case of Boxers, T-cell lymphomas can account up to 87% of all cases.<sup>62</sup> The distribution of lymphoma immunophenotype in mixed breed dogs are for B-cell and T-cell lymphomas 70% and 30%, respectively, percentages comparable to the observed when all purebred dogs are considered as a single group.<sup>58</sup>

The presumptive association of certain breeds and disease occurrence, is also associated to reports of familiar<sup>63</sup> or clusters<sup>64</sup> developing lymphoma, what might suggest heritable genetic

aberrations, predisposing the mentioned breeds to develop NHL and/or specific phenotypes.<sup>65</sup>

Sex seems to be determinant in determining risk, with female sex associated with lower risk,<sup>52,66,67</sup> possibly due to a protective role of endogenous oestrogens, however contradictory report exists<sup>61</sup> and this hypothesis needs corroboration. The removal of gonads (neutering and spaying) has been associated with increase incidence of tumours, namely lymphoma.<sup>68</sup> Neutered male Golden Retrievers<sup>69</sup> showed a three times increased risk than intact males and in Australian dogs, males and both, neutered males and females, showed an increased lymphoma-risk.<sup>70</sup> The presence of Luteinizing Hormone (LH) receptors in normal and neoplastic lymphocytes and the increased constant exposition to LH due to the absence of negative feedback from testosterone or oestrogens on gonadectomised dogs, can trigger lymphoma by increasing cell proliferation through LH receptors.<sup>71</sup>

### 1.2.3 Aetiology

The causes of canine non-Hodgkin lymphoma remain largely unknown. Numerous studies have been conducted in order to identify the possible causative agents of lymphomagenesis in dogs, but cL's aetiology is largely unknown and probably multifactorial.<sup>11</sup> The first study reporting a possible aetiology dates from 1945 and is attributed to Bloom and Meyer, that associated cL with putative conditions such trauma or parasitic infestations as doxmiiasis.<sup>43</sup> Understanding and identifying the mechanisms that can contribute to disease development may help the signalization of risk populations, adoption of respective preventive measures and screening tests or, even, the development of new therapeutic approaches.

#### 1.2.3.1 Environmental factors

A controversial case control study, conducted in 1991, by Hayes and co-workers, indicated that dogs living with humans that applied herbicides in their households, particularly 2,4-dichlorophenoxyacetic acid (2,4-D), had a significant higher risk of developing lymphoma, when compared with two control groups (one composed of cancer free dogs and the other with dogs with cancer except lymphoma and bladder tumour). The same study revealed that the risk of developing canine malignant lymphoma rose to a twofold excess with four or more yearly owner 2,4-D applications.<sup>72</sup> Another study, evaluating N-ras mutational activation in 2,4-D exposed dogs with lymphoma, concluded that N-ras mutations were infrequent.<sup>73</sup> Further studies confirmed that exposure to 2,4-D in dogs is not associated with malignant lymphoid transformation by mechanisms involving N-ras mutation.<sup>74,75</sup> A deeper look at the

environmental causes has continued and a couple of other studies of risk evaluation after environmental pollutants exposure have been undertaken. In 2001, in Italy, Gavazza and co-workers studied 101 cases of dogs diagnosed in a teaching institution in a two-year period (1996-98) and reported a positive and independent relationship between the development of lymphoma in dogs living in industrial areas and co-living with owner's that use paints and organic solvents.<sup>76</sup> Also in Italy, researchers from a veterinary referral hospital compared, between 2003 and 2008, canine and feline cancer risk in Naples (considered a low risk area) and some Naples satellite cities with reported history of illegal waste dumping (high risk areas). An increased risk for cancer development and specifically, a 2.39-fold increased risk of lymphoma were observed in dogs living in the satellite cities.<sup>77</sup> In Porto, Portugal, the geographical distribution of human and canine Age-Standardized Risk (ASR) was well correlated ( $r=0,664$ ,  $p<0,05$ ), with the highest values for human and canine ASR detected in the same urban municipalities of Porto, Maia and Matosinhos, probably due to the same exposure to environmental factors.<sup>66</sup>

Takashima and co-workers (2012), evaluated the possible association between the use of flea and tick control products and lawn-care chemicals and the risk of canine lymphoma development and concluded that the use of lawn care products was associated with a 70% risk increase of dogs developing the disease. No evidence of association was found between the use of flea and tick control products and risk for developing cL.<sup>78</sup>

Exposure to tobacco smoke has been related to lymphoma development in Humans<sup>79</sup> and no data exists for dogs; however, it seems that tobacco exposure can increase lymphoma aggressiveness in dogs, associated with expression of higher Ki67 index.<sup>80</sup>

In the United Kingdom, the diagnosis of cL at first opinion veterinary practices had a weak evidence of spatial association with clustering around London and the south-west of England and the use of herbicides.<sup>81</sup>

### *1.2.3.2 Genome and molecular aberrations*

It is consensual that malignant transformation of cells needs the cumulative addition and interaction of several genetic abnormalities, at a sub-cellular level, specially changes in chromosome number and/or structure.

The perception that humans and dogs have relative similar genome homology has increased as new genetic tools enabled a deeper insight and became largely available.<sup>82,83</sup> These technologies are currently applied to understand the molecular and genetic framework of mechanisms underlying lymphomagenesis.

Canine non-hodgkin lymphoma has less genomic instability than its human counterpart as has been demonstrated by the use of comparative genomic hybridization. This highlighted chromosomes 13 and 31, corresponding, respectively, to human chromosomes 8 and 21, as the most affected by copy number aberrations; aneuploidy and trisomy of chromosome 13 can contribute to 75% and 25%, respectively, to karyotype abnormalities in cL.<sup>84</sup> In a cohort study with 61 cNHL, Hahn and co-workers (1994), using conventional cytogenetic techniques, found several chromosomal aberrations in dogs, mainly reciprocal chromosomal translocation resulting from the centric fusion of two acrocentric chromosomes; these authors also identified the presence of chromosome 13 trisomy. These aberrations were significantly associated with first remission length. Also, survival time, was significantly longer in 25% of dogs with chromosome 13 trisomy as the primary chromosomal aberration than in the 75% with other primary chromosomal aberrations.<sup>85</sup> Treatment naïve DLBCL can show copy number aberrations associated with different genomic imbalances consisting of chromosomal gains and losses. Gains in chromosome 13 (chr13) were significantly correlated with WHO clinical stage and specific regions of losses and gains were significantly associated to duration of remission. Losses involving locus for Immunoglobulin Kappa (IGK), Immunoglobulin Lambda (IGL), and Immunoglobulin Heavy (IGH) chains were always found, and gains along the length of chr13 and chr31 are often observed.<sup>86</sup> Lymph nodes can harbour a proportion of aberrant cells consistently higher, than the peripheral blood, being trisomy of canine chromosome 13 the most common aberration present (56%) in nodal malignant cells. Canine chr13 aberrations are important from the comparative point of view, since chr13 shows high homology to regions on the human chromosomes 4 and 8, where the proto-oncogenes c-kit and c-myc are located.<sup>87</sup> DLBCL in the dog, shares the same gene fusion Immunoglobulin Kappa Chain-CyclinD3 (IGK-CCND3) as its human counterpart.<sup>88</sup> Nevertheless, to the date when compared with hNHL, cNHL is associated with more limited genomic instability and has no evidence for Cyclin-Dependent Kinase Inhibitor 2A/B (CDKN2A/B) gene deletion in B-cell type, supporting lymphomagenesis.<sup>89</sup> In Golden Retrievers, a high risk breed for cancer development, a preliminary study suggested that individual susceptibility risk to develop lymphoma, can be associated with deficiencies in heritable factors related to Deoxyribonucleic Acid (DNA) repair capabilities promoting and increasing sensitivity to chromosomal damage.<sup>90</sup> Monosomy X has also been reported in a B cell canine lymphoma cell line, as the sole clonal chromosomal abnormality.<sup>91</sup> Genomic hypomethylation can be present up to 69% of the cases, increasing genomic desregulation<sup>92</sup> and apparently with extension to non-neoplastic circulation leukocytes.<sup>93</sup> Hypermethylation of p16 tumour suppressor gene through its Cytosine-Guanine (CpG) island

was identified in canine lymphoma derived cell lines.<sup>94</sup> In the particular case of gastrointestinal lymphoma in dogs, genome-wide DNA methylation analysis, revealed a large number (383-3,054) of hypermethylated CpG sites, that could be involved in lymphomagenesis.<sup>95</sup> A mean methylation level of Tissue Factor Pathway Inhibitor-2 (TFPI-2) gene with over three-fold higher, was reported to be present in dogs with lymphoma compared with healthy controls, providing the first evidence of age-associated epigenetic modifications in dogs.<sup>96</sup>

In T-cell lymphomas, evidence of mutations on p53 tumour suppressor gene,<sup>97</sup> loss of chromosome 11, deletion of p16 gene and Retinoblastoma protein (Rb) phosphorylation in Cyclin Dependent Kinase 4 (CDK4) sites, have been described. This Rb phosphorylation was also identified in two types of B cell tumours: DLBCL and Burkitt's Lymphoma (BL). In these particular neoplasia, Rb phosphorylation seemed to be associated with cell-myelocytomatosis gene overexpression.<sup>98,99</sup>

The Nuclear Factor Kappa-light-chain of B-cell (NF- $\kappa$ B) belongs to a family of transcription factors that are key regulators of B-cell development, proliferation and survival. Human and canine DLBCLs are characterized by constitutive canonical NF- $\kappa$ B activity that promotes lymphomagenesis and treatment resistance via overexpression of anti-apoptotic NF- $\kappa$ B target.<sup>100,101</sup> More than 30% of B-cell cL can contain one or more somatic Tumour Necrosis Factor Receptor-Associated Factor 3 (TRAF3) mutation which codes a negative regulator of NF - $\kappa$ B.<sup>102</sup>

Mudaliar and co-workers (2013) through the analysis of differentially expressed probe sets, confirmed these shared molecular signatures in canine and human DLBCL, reporting the enrichment of the NF- $\kappa$ B signalling pathway in both species, with potential therapeutic implications.<sup>103</sup>

Alterations on the B-cell Lymphoma 2 (Bcl-2) family of proteins, the key regulators of apoptosis, is an hallmark of B-cell lymphoma in humans.<sup>104</sup> The expression of Bcl2 protein, that is a key anti-apoptotic molecule and, Bcl2 Associated X Protein (BAX) another Bcl-2 family molecule with pro-apoptotic functions, was assessed in a small population of dogs with multicentric lymphoma. The Bcl-2/BAX ratio was significant higher in T-cell when compared with healthy controls. Dogs with B-cell lymphoma had similar values for Bcl-2/BAX when compared with healthy controls.<sup>105</sup> This higher ratio Bcl-2/BAX can be one reason to explain worse response to therapy in canine multicentric T-cell lymphoma, comparing with the B counterpart, and needs further evaluation, specially because it can be another potential therapeutic target.<sup>104</sup>

T-cell lymphomas have an important mutational landscape, that includes mutations in genes involved in several signalling pathways such as: 1) MET, KDR, STK11, BRAF. MYC and TP53

missense mutation<sup>106</sup>; 2) down regulation of PTEN signalling and upregulation of MTOR-PI3K-ATK<sup>107</sup>; 3) absence of CD45 transcription, specifically in T Zone Lymphoma(TZL)<sup>108</sup> leading to loss of expression of CD45.<sup>109</sup>

Aberrations in the Fragile Histidine Triad (FHIT) gene and its expression, were identified in five canine lymphoma cell lines, and are considered possible important factor in promoting canine lymphomagenesis.<sup>110</sup>

Other cytogenetic abnormalities observed in naturally occurring canine hematopoietic tumours are comparable to characteristic features of the corresponding human disease. Chronic Myelocytic Leukaemia (CML) in dogs presents the high-molecular-weight BCR-ABL fusion protein, demonstrating a Myc/IgH-like translocation in cases of cNHL; simultaneously, Myc is constitutively expressed in 78% of the cDLBCL. In the cNHL subtypes that don't have chr13 aberration, Myc expression was only seen in Burkitt lymphoma cases.<sup>82</sup>

Dog's malignant B and T-cells can show increased messenger Ribonucleic Acid (mRNA) expression for multidrug resistance genes ABCB1 and ABCG2.<sup>111</sup> MicroRNA (miRNA) expression patterns can also help differentiating neoplastic from inflammatory infiltrates in canine intestinal T-cell lymphomas, with the neoplastic cells having an expression pattern with down regulation of the tumour-suppressing microRNAs miR-194, miR-192, miR-141 and miR-203, and up-regulation of oncogenic microRNAs, from the miR-106a~363 cluster.<sup>112</sup> In Marginal Zone Lymphoma (MZL) and Follicular Lymphoma (FL), transcriptome profiling revealed shared biological processes, on B-Cell Receptors (BCR) and Toll-Like Receptors (TLR) signalling pathways: FL showing an enrichment of E2F targets, whereas MZL were characterized by MYC driven transcriptional activation signatures. FL showed a distinctive loss on chromosomes 1 and 24, and MZL presented multiple recurrent gains on chr13; The distribution of methylation peaks was similar between the two histotypes.<sup>113</sup>

The knowledge of genetic and chromosomal abnormalities in cNHL is still in an early stage, when compared with the human field. Nevertheless, it has been showing a promise field to better understand the molecular and genetic mechanisms that trigger and sustain lymphomagenesis, to recognise similar genetic pathways in human and canine lymphomas and, when possible, identify specific abnormalities that can be associated with respective phenotypes and morphological entities, defining prognosis, and possible therapeutic targets.<sup>100</sup>

### *1.2.3.3 Immune Status*

The immune system plays a fundamental role in identifying and eliminating tumour cells by means of immune surveillance. Tumour cells that escape immune surveillance through a

process designated by immunoediting, by which can not only evade anti-tumour immunity but also, lead to an immune deregulation, stimulating immunological conditions that promote tumour progression and suppress anti-tumour adaptive and innate responses.<sup>114</sup> Description of impaired immune function in dogs affected by lymphoma dates back to 1975 by Owen and colleagues.<sup>114</sup> The concomitant occurrence of lymphoma and various immune-mediated diseases such as lupus and pemphigus foliaceus,<sup>115</sup> polyarthritis, haemolytic anaemia and thrombocytopenia; only the dogs with the later auto-immune disorder showed to have an increased odds ratio (5,61) to develop lymphoma when compared with the general population.<sup>116</sup> The treatment with immune suppressant drugs, such as cyclosporine, has also been associated with the development of lymphoma.<sup>117</sup> A possible association between Canine Atopic Dermatitis (CAD) and the development of Mycosis Fungoides (MF) was suggested by Santoro and colleagues (2007), describing an odds of developing MF of 12 times higher in dogs with CAD.<sup>118</sup>

Intestinal microbiota has a significant importance in the immune status, through a constant communication between *microbiota* and immune system in tolerance and activation scenarios. There is some evidence that disruption of this equilibrium contributes to lymphomagenesis in humans.<sup>119</sup> In dogs with intestinal lymphoma, the presence of significant increases in organisms belonging to the Eubacteriaceae and *Streptococcus spp.* and lower abundance of *Faecalibacterium spp.*, *Fusobacterium spp.* and *Turicibacter spp.*, revealed a significant higher dysbiosis index, when compared to healthy individuals.<sup>120,121</sup>

Lymphoma, doesn't seem to affect the oxidative burst and phagocytic activity of neutrophils, that maintain a good bactericidal activity.<sup>122</sup> After chemotherapy initiation, the oxidative burst decreases over time, whereas phagocytic capacity improves, comparing with baseline and to healthy controls.<sup>123</sup> Also, lymphoma specific treatments do not seem to compromise immune responses.<sup>124,125</sup>

Immune checkpoint molecules such as Programmed Cell Death 1 (PD-1) and its ligand (PD-L1) are important to maintain T-cell self-tolerance. Tumour cells frequently express high levels of PD-L1, which results in immune paralysis of T-cells and escape of tumour cells from T-cell-mediated death.<sup>126</sup> Malignant B-cells have increased expression of PD-L1, when compared to normal B cells.<sup>127</sup> In the case of T cell lymphomas, tumour cells and normal T-cells both showed low to negative expression of PD-1 and PD-L1.<sup>128</sup>

The emergence of cutaneous demodicosis<sup>129,130</sup> in dogs affected with TZL, brings out the discussion of TZL development in or causing an immunosuppression environment.<sup>131,132</sup>

The exact mechanism by which chronic inflammatory processes induce lymphomagenesis is still unclear, but persistent immunosuppression<sup>117</sup>, epigenetic events<sup>133</sup> and relevant

association with immunosenescence<sup>134</sup> have all been associated with lymphoma development.

#### *1.2.3.4 Infectious diseases*

Infections are associated with inflammation and immune deregulation, both processes that can lead to tumorigenesis.<sup>29</sup> There are sporadic reports of dogs with concomitant infectious diseases such as *Ehrlichia canis*<sup>135,136</sup>, *Bartonella spp.*<sup>35</sup>, *Leishmania spp.*<sup>37,36</sup> or *Heterobilharzia americana*<sup>137</sup> and lymphoma. Retroviral density particles and reverse transcriptase activity were identified in culture supernatants from canine lymphoma cell lines.<sup>138</sup> The serological evidence of exposure to Epstein-Barr Virus (EBV) or an EBV-like virus in dogs, was detected in dog sera in United Kingdom and United States, but no evidence was found of EBV in canine peripheral blood mononuclear cells.<sup>139</sup> Huang and co-workers (2012) reported the detection of specific IgG for anti-EBV capsid antigens in both healthy and lymphoma-affected dogs. They also demonstrated the presence of EBV-like viral DNA and RNA sequences and Latent Membrane Protein-1 (LMP-1) in malignant lymph nodes and the presence of herpes virus particles by electron microscopy. This study strongly supports the hypothesis that dogs can be naturally infected with an EBV-like gamma herpes virus that may contribute to lymphomagenesis.<sup>140</sup> A more recent study performed with the author's collaboration did not confirm the molecular presence of gamma herpes virus in a vast series case of canine lymphoma,<sup>141</sup> sustaining uncertainty about this association.

### **1.3. Disease classification**

#### *1.3.1 Classification schemes*

Several classification systems that have been applied to lymphoproliferative disorders along the years have relied in the current knowledge of the scientific community at the time. Canine lymphoma classification can be considered as a continuous process that follows the constant scientific achievements and subsequent deepening of knowledge on the disease.

The first report of lymphoid neoplasms dates from early 19th century, when Sir Thomas Hodgkin described the first round cell neoplasia that is still designated by his name: Hodgkin's lymphoma. This happened about ten years before Virchow's leukaemia description in 1845 and later lymphoma in 1863.<sup>142</sup> The first and very rudimentary form of lymphoma classification was elaborated in 1942 using a pre-classification American scheme designated the "Registry". In this first attempt, seven categories were identified and the cytological

characteristics were the base of the categorization.<sup>143</sup> Twenty years later in 1966, Henry Rappaport, based on Ashoff's theory that lymphocytes derive from the reticule-endothelial system and all blood and connective tissue cells would derive from an undifferentiated mesenchymal cell,<sup>142</sup> proposed in 1966, an algorithm to aggregate lymphomas based only on cell characteristics, taking in special attention the nodular and diffuse patterns.<sup>144</sup> It was the first time that the scientific community recognised the lymphomas types as distinct and distinguishable from one another. Despite being widely accepted, because its premises were based on incorrect concepts, it soon showed inadequate for clinical and fundamental research. Rappaport's believed that neoplastic lymphocytes could derive from histiocytes or a mixture of lymphocytes and histiocytes and it soon was proved to be wrong.<sup>145</sup> Also, lymphomas were regarded as unique immutable conditions, from lymphocytic, hystiocytic or "mixed cell" derivation. Along it contemplated also the clear distinctions between Hodgkin and non-hodgkin lymphoma.<sup>146</sup>

The increasing development obtained in research in areas like microscopy, cellular biology and immunology brought new insights for understanding normal and neoplastic lymphocyte biology, as well as new conceptual orientations. The use of antibodies and the identification of different lineages of morphologically identical cells, emphasized the need of a new stratification, based not only on the morphology, but also on the immunophenotype. As a result, two relative different systems came up. In 1981, in Kiel, Germany, Karl Lennert conceived the so-called "Kiel classification", having in account morphologic and immunophenotype characteristics.<sup>147</sup> At the same time but in the United States of America (USA), Robert Lukes and Robert Collins, attempted to classify lymphomas, ignoring morphological pattern and assuming that only cell type (B or T) was determinant in clinical progression.<sup>142</sup> Lukes and Collins described the entity "follicular centre cell", because they considered the follicular centre as the site of B-cell transformation.<sup>148</sup> These concepts make Lukes-Collins classification mainly based in functional characteristics, omitting subdivisions concerning malignancy grade. Kiel classification scheme was anchored in two principles: i) correlation between each lymphoma category and its non-neoplastic B or T-cell homologous; at the same time it provides a certain information about the grade of malignancy, based on the mitotic index and quantity of blasts present and ii) each tumour corresponds to a blocked (permanent or transitory) stage of maturation, during the physiologic lymphoid evolution.<sup>142,145</sup> Concerning prognostic information, the Kiel scheme made a differentiation of low grade, suffixed as "-cytic or -cytoid" and high grade, suffixed as "-blastic" tumours. Kiel's proposal was widely accepted in Europe, in part due to European tradition of a pathophysiologic disease approach.<sup>145</sup> Despite its acceptance, Kiel's classification was subjected to revision and updating in 1988 and later in 1992, mainly due to the development

of monoclonal antibodies that allowed the introduction of more immunological criteria. Kiel's updated classification has benefited with the emergence of specific cell lineage markers, highlighting the fact that some tumours with B-cell morphology were, in fact, of T cell phenotype and that T immunophenotype was a very important independent negative prognostic factor.<sup>149</sup> The ability to identify the cell lineage of lymphoid cells has shaped the modern classification of lymphomas, and is a starting point for diagnosis.<sup>150</sup> Until then, T lymphocyte derived lymphomas were poorly documented.<sup>15,142</sup> Kiel's classification became the standard lymphoma classification scheme in Europe for human lymphoma and easily applicable to cL,<sup>151</sup> while in the USA neither Kiel nor the Lukes-Collins's scheme were popular, promoting an heterogeneous classification approach and communication difficulties either in or between the clinical and pathology professionals.<sup>145,15</sup> The United States National Cancer Institute (NCI), in response to the various negative reactions of this lack of standardising and communication, promoted an international collaborative work, in 1982, known as the NCI-Working Formulation for Clinical Usage (NCI-WF). The NCI-WF became the gold standard scheme in the USA, contrary to the Kiel's classification used in Europe. The descriptive and classification confusion couldn't last for long. A new decade was starting and, in 1991, due to the concerted efforts of 18 european and american hemato-pathologists the International Lymphoma Study Group (ILSG) was created. The ILSG elaborated then a new classification scheme, which was further revised, resulting in the so-called Revised European American Lymphoma (REAL) Classification.<sup>152</sup> This work was of tremendous importance, being the most frequently quoted medical publication on the last decade of the 20th century.<sup>145</sup>

This success was due, mainly, to the definition of lymphoma subtypes which could be recognized by morphologic, immunologic and genetic characteristics, describing "real" entities, distinguishing between B or T-cells' derived cancers and, their respective precursors and mature cells localized in the peripheral organs of the immune system. The name Hodgkin's disease was changed to Hodgkin's lymphoma, due to the proven lymphoid origin. Two other very important achievements in the REAL scheme were the elimination of the malignancy "grades", once it was assumed that disease behaviour can be associated to individual patient factors, that might vary between patients in the same disease category, leading to the implementation of a more personalized therapy.<sup>145,152</sup> In 1994 a committee from the WHO decided that REAL classification represented the model for the updated organization of lymphomas and its methodology was extended to all neoplastic conditions derived from the hematopoietic tissues, giving birth to the third edition of the WHO Classification of Hematopoietic Tumours of Lymphoid Tissues.<sup>152,153</sup> More than 150 years after Rudolf Virchow's description of leukaemia, the fourth edition of the WHO Classification

of Tumours of Haematopoietic and Lymphoid Tissues<sup>154</sup>, also known as the Blue Book, is released. In this classification is included the description of 103 types of tumours from the hematopoietic and lymphoid tissues based in the REAL principles, so the importance is to define “real” diseases that can be recognised by pathologists and clinicians. The Blue Book constitutes one of the major references with up-to-date scientific information on diagnosis and management of hematopoietic and lymphoid neoplasia in humans. The WHO lymphoma classification has shown to be reproducible and the defined diseases are clinically distinctive, facilitating clinical and translational studies<sup>145</sup> being updated recently in 2016.<sup>155</sup> This document raised the importance of a multi diagnostic tool approach (flow cytometry, genetic, molecular, chromosome abnormalities, morphology and immunohistochemistry) to fulfil a classification based on clinical, morphologic, genetic and immunophenotype features.

With the advent of available high-throughput methods such as comparative genomic hybridization, single nucleotide polymorphism arrays, gene expression profiling, microRNA and tissue microarrays, more knowledge will be achieved regarding early stages of lymphomagenesis, refining diagnostic criteria, to identify progression and resistance to therapy.<sup>150</sup>

A correct classification must correspond to the current knowledge on lymphomagenesis, having a key role in developing effective therapies. This urgency on knowledge is crucial, if considering that lymphoma will become the most common form of cancer within the next two decades.<sup>145</sup>

### *1.3.2 Canine lymphoma classification*

Three years after the first attempt to classify human lymphomas,<sup>143</sup> the same attempt was made for dogs and that was defined as the “importance of the comparative aspects of leukaemia in lower animals” *sic*.<sup>143</sup> Hodgkin’s disease is rare in animals and there is only one case report of a Hodgkin-like lymphoma identified in a dog.<sup>156</sup>

Historically, the first descriptions of lymphoma classification in dogs used Rappaport scheme and dates from 1976.<sup>157</sup> The Rappaport, and Working Formulation, classifications had less predicting value for prognosis, when applicable to dogs, since all tumours in animals were classified mostly as diffuse, high grade with associated hystiocitic cellular component.<sup>158</sup> The Lukes–Collins scheme was easily and successfully applied to canine lymphomas, mainly due to the close morphologic similarity between canine and human normal lymph nodes.<sup>142</sup> The Kiel classification system was applicable mainly to nodal pathology; advances in immunologic concepts and application of immunohistochemistry indicated the need for update all of the previous systems. Using the National Cancer Institute Working Formulation (NCI-WF) that

was less oriented for the cellular morphology and more towards the clinical outcome, Carter and co-workers<sup>159</sup> have demonstrated the applicability of NCI-Working Formulation classification to cL. The authors admit the limitation of the Working Formulation to predict survival, as it predicts in humans, since in dogs, the owners can decide sooner the end of life of the animal, biasing results. The Working Formulation became widely used in North America and it was easily adaptable to canine lymphomas,<sup>149</sup> whereas the Kiel system became widely used in Europe for both humans and animals.<sup>144,149,160</sup>

B-cell immunophenotype is the most common in cL, accounting for up to two thirds of subtypes, T-cell lymphomas, contribute to up to 30% of all cases (with origin in T and NK cells) and, rarely, non-B/ non-T tumours.<sup>15,145,141</sup> This percentage it's not always applicable since this distribution can vary among dog breeds, as previously described.

Nowadays, the revised WHO system of classification for hematopoietic and lymphoid tissues<sup>15</sup> is the state-of-art classification scheme and internationally accepted in veterinary medicine (Table 1); nevertheless, in veterinary literature the updated Kiel classification is also, along with the WHO classification, commonly used,<sup>17</sup> being the first mainly applicable to cytology specimens.<sup>144</sup>

#### *1.3.2.1 Anatomic classification*

The relative frequency of particular affected anatomic sites, together with the respective associated clinical characteristics, led to the adoption of a practical classification system that is commonly used among clinicians and pathologists: the anatomical classification.

The most common anatomical presentation in dogs is the multicentric form, where several nodal and extra-nodal lesions are present, accounting for about 80% of all cases.<sup>162</sup> Several other locations are distinguished but accounting for less than 10% in frequency each, such as the alimentary, cutaneous, or other extra-nodal forms.<sup>11</sup> In the multicentric form dogs present enlarged, sometimes symmetric/asymmetric and bilateral, non painful, fast growing, mobile or fixed, peripheral lymph nodes (submandibular, cervical, pre-scapular, axillar, inguinal and popliteal) associated or not, with abdominal distension due to mesenteric lymph nodes, hepatic and/or splenic enlargement due to infiltration.<sup>11</sup> Affected nodes can be as few as one node, few nodes, in the same or opposite sides of diaphragm or generalized adenomegaly,<sup>11,163</sup> Hepatosplenic involvement can be, by itself, a particular form of anatomical localization associated with a specific prognosis and clinical course or being, most commonly, an extension of the multicentric presentation.<sup>164</sup>

**Table 1:** Classification of canine lymphoma according to WHO subtypes. (Adapted from Valli VE, et al. 2011)<sup>15</sup>

<b>B-cell neoplasia</b>	<b>T &amp; NK neoplasia</b>
Immature	
Precursor B lymphoblastic leukaemia/lymphoma	Precursor T-cell neoplasm
Precursor T lymphoblastic Lymphoma/leukaemia	
Mature	
B-cell chronic lymphocytic leukaemia/pro-lymphocytic Leukaemia/ Small lymphocytic lymphoma	T-cell pro-lymphocytic leukaemia
Pro-lymphocytic leukaemia	Large granular lymphocyte leukaemia
Lymphoplasmacytic lymphoma	Aggressive natural killer cell leukaemia
Splenic marginal zone B-cell lymphoma	Peripheral T-cell lymphomas, unspecified
Plasma cell myeloma/ Plasmacytoma	Adult T-cell lymphoma/leukaemia
Extra-nodal marginal zone B cell lymphoma of mucosa-associated lymphoid tissue type (MALT)	Intestinal T-cell lymphoma (+enteropathy associated)
Nodal marginal zone lymphoma	Hepatosplenic gamma-delta
Follicular lymphoma	Subcutaneous panniculitis-like T-cell lymphoma
Mantle cell lymphoma	Mycosis fungoides/Sézary syndrome
Diffuse large B-cell lymphoma	Anaplastic large cell lymphoma, T and null cell primary cutaneous type
Mediastinal large B-cell lymphoma	Peripheral T-cell lymphoma not-otherwise-specified
Burkitt's lymphoma/Burkitt's cell leukemia	Angioimmunoblastic T-cell lymphoma
Provisional entity: high-grade B cell lymphoma Burkitt's-like	Angiocentric T-cell lymphoma
Primary effusion lymphoma	

This termed "anatomical classification" is helpful in a clinical environment because clinicians can exchange information on basis of presentation, clinical course and therapeutic decisions, as these anatomical presentations have been linked with expected clinical contexts and prognosis.<sup>164-168</sup>

#### 1.3.2.1.1 Multicentric disease

In the great majority of the cases, the disease presents as painless enlargement of several peripheral lymph nodes.<sup>160</sup> This multicentric form of disease in dogs accounts for around 75-80% of the total cases, being mostly 95% of cases from B immunophenotype.<sup>11,44</sup> The mediastinal, intestinal, splenic, hepatic, renal, central nervous system, ocular and cutaneous forms have lower reported incidences.<sup>13</sup>

Despite its, mainly, aggressive nature, patients tend to have mild to no clinical signs at initial presentation. When disease progresses, mild to severe symptoms can be present depending on WHO stage and affected organs.<sup>13</sup>

#### 1.3.2.1.2 Alimentary form

High-grade Gastrointestinal (GI) lymphoma accounts for approximately 5–7% of all canine lymphomas and are mostly of a T-cell immunophenotype revealing epitheliotropism resembling Human Enteropathy-Associated T-cell lymphomas Type 1 (hEATCL type 1)<sup>169</sup> and it can be constituted by large or small cells, rarely mixed, with different cytological characteristics. The presence of co-expression of CD20 on malignant T-cell can be observed sporadically, associated with a clonal rearrangement for TCR, but its true value for prognosis and response to treatment is unknown.<sup>170</sup> Despite neoplastic lymphocytes resembling the ones seen in hEATCL morphologically there are remarkable differences. The presence of medium sized monomorphic lymphocytes is rarely seen in dogs and a considerable presence of inflammatory infiltration is existent, what is rarely seen in hEATL type II. Small cell lymphomas have an increased infiltration of plasma cells and do not express CD56 that is expressed by 12% of the large cell tumours.<sup>171</sup>

Primary gastrointestinal lymphoma in dogs occurs over a wide range of ages and breeds and increased male risk is under discussion. Reported breeds with increased risk for development of intestinal T-cell lymphoma are Shiba dogs, German shepherds, Cairn and Boston terriers, Papillons, Pugs and Maltese.<sup>172</sup> Primary GI lymphoma normally does not affect the peripheral lymph nodes or the spleen and affects by order of decreasing frequency, the small intestine, stomach and colon, nevertheless the disease presents as multifocal all over the GI tract. The neoplastic lesions usually disrupt normal gut layers and may involve several segments of the intestine. The disease can progress to regional lymph nodes, liver and spleen. Clinical signs are non-specific and common to other gastrointestinal diseases with prognosis being poor for high-grade forms.<sup>11,173</sup>

Multidrug chemotherapy treatment of diffuse intermediate to high grade forms are associated with poor treatment response and survival, with median survival times of a few months.<sup>166,173–175</sup> The most aggressive subtype is the anaplastic lymphoma (CD3-CD30<sup>+</sup>) with median survival time of 5 days.<sup>169</sup>

Low-grade, indolent, gastrointestinal lymphoma in dogs tends to be T-cell phenotype, and associated with the same unspecific gastrointestinal clinical signs. No sex or age predisposition are described and Boxers and Sharpei breed dogs appear described with higher numbers in reports.<sup>173,174</sup> Usually, it shows good clinical response to treatment and

prognosis.<sup>176,177</sup> A rare form of colorectal lymphoma has been recently described in three dogs. The disease presented as solitary nodes in 2/3 dogs and in one dog as multiple local lesions. Surgery led to long complete remissions in the dogs with unique lesion and disease progressed to systemic disease in the dogs with multiple nodules, responding favourably to CHOP chemotherapy, with a complete remission for more than 2 years. This type of follicular colorectal lymphoma is characterized by B-cell immunophenotype and BCL2<sup>+</sup>, BCL6<sup>+</sup>, CD3<sup>-</sup>, CD5<sup>-</sup>, CyclinD1<sup>-</sup>.<sup>178</sup>

#### 1.3.2.1.3 Cutaneous lymphoma

Canine Cutaneous Primary Lymphoma (cCPL) represents 3-8% of the cL cases and affects, mostly, older animals, with no sex or breed predisposition, although, Bichon Frisé and the Bouvier des Flandres can be overrepresented.<sup>179,180</sup>

Tumour cells can invade oral and mucocutaneous areas, on the epidermis, superficial dermis and peri-adnexal tissues.<sup>181</sup> Cell phenotype is mostly T: CD3<sup>+</sup> and CD4<sup>-</sup>/CD8<sup>+</sup>, with frequent expression of the gamma-delta T-cell Receptor (TCR $\gamma\delta$ )<sup>180</sup> and abnormal expression of retinoid receptors.<sup>182</sup> In pagetoid reticulosis, lymphocytes consistently express TCR $\gamma\delta$  while some other variants like TCR $\alpha\beta$ <sup>+</sup> can be as well present.<sup>183</sup> When epitheliotropism is present Type-1 cytotoxic T-cells are the main representative population<sup>179</sup> assuming the form of a mushroom and designated by Mycosis Fungoides (MF). Despite being of T-cell phenotype, CD20 expression is not rare and can be present in more than 50% of cases, making this markers (CD3, CD4, CD8 and CD20) unsuitable to differentiate between B and T cell cutaneous lymphomas.<sup>184</sup> Dogs affected by MF can have different lesion types, such as generalized “exfoliative erythroderma” (comprising generalized erythema, pruritus and scaling), muco-cutaneous lesions with ulcers, erosion and/or depigmentation, solitary or several cutaneous nodules or plaques throughout the skin, or infiltrative oral mucosa lesions. Vesiculobullous lesions are rarely present in epitheliotropic cL.<sup>185</sup> A progressive form of cCPL, with a leukemic phase and where the neoplastic cells have a characteristic appearance with cerebriform nuclei (Sézary cells) is designated as Sézary syndrome, it is very rare in dogs with only one case described in literature.<sup>186</sup>

Affected animals, apart from the cutaneous lesions, can show other clinical signs, such as polyuria/polydipsia caused by hypercalcemia, adenomegaly, multi-organ involvement and associated systemic signs due to tumour progression.<sup>181</sup> Despite the inflammatory-like presentation, cCPL doesn't seem to express significantly the pro-inflammatory protein Cyclooxygenase-2 (COX-2).<sup>187,188</sup>

Treatment response is poor, radiation therapy for muco-cutaneous oral forms induces durable clinical response in more than half of the patients<sup>189</sup> and the alkylant drug Lomustine, in general, seems to be the best choice, inducing a complete but short remission in one third of the treated dogs.<sup>190</sup> More recently, the utilisation of isotretinoin and interferon- $\alpha$  in 2 cases of cCPL was described and associated with better outcomes than Lomustine and prednisolone.<sup>191</sup>

#### 1.3.2.1.4 Mediastinal lymphoma

Mediastinal forms can be primary (5% of cases) or secondary to multicentric disease, namely on T-cell phenotype CD4<sup>+</sup>/CD8<sup>+</sup>,<sup>183</sup> where mediastinal involvement of multicentric disease can happen in more than 50% of the cases. It can be of B or T-cell immunophenotype, and the lately in the lymphoblastic form can comprise up to 96,6% of the T-cell cases.<sup>192</sup> Young to middle age animals are overrepresented,<sup>13,192,193</sup> and despite affecting several breeds, Golden Retrievers, Labradors, non pure-breed and Boxers may be more prone to development.<sup>192</sup> Disease manifestation is characterized by the presence of enlarged cranial mediastinal lymph nodes, associated or not with thymic involvement.

Affected animals can develop lethargy, anorexia, polyuria-polydipsia, coughing, dyspnoea or tachypnea and vomiting. Pleural effusion and paraneoplastic hypercalcemia, can be present in 45,2% and 67,5% of the cases, respectively; when present, hypercalcemia appears not to be a negative prognostic factor, but the presence of pleural effusion decreases overall survival.<sup>192</sup> Head and neck oedema can also be present, due to superior vena cava syndrome, if the tumour mass compromises venous return to the right heart. Dogs affected by multicentric forms can have, in about 20% of cases, secondary craneo-mediastinal node involvement.<sup>194</sup> When treated with multi-agent protocol CHOP, Progression Free Survival (PFS) and Overall survival (OS) are 133 and 183 days, respectively.<sup>192</sup>

#### 1.3.2.1.5 Hepatosplenic lymphoma

Hepatosplenic primary lymphoma is a rare aggressive disease, generally not associated with other lymphatic tissue involvement, apart from bone marrow. It is normally of  $\gamma\delta$  T-cell origin, as occurs in humans<sup>164</sup> and can highly express MHCII and CD4.<sup>183</sup> Two different forms of hepatic involvement are described, depending on the pattern of neoplastic cells distribution: 1) along hepatosplenic sinusoids-designated Hepatosplenic T-cell lymphoma-similar to the Human counterpart and 2) only and along hepatic cords, designated

Hepatocytotropic lymphoma.<sup>195</sup> Neoplastic lymphocytes in hepatosplenic lymphoma are CD11d<sup>+</sup>, CD4<sup>-</sup>/CD8<sup>+</sup> and CD3<sup>+</sup>/TCR $\alpha\beta$ <sup>-</sup>/TCR $\gamma\delta$ <sup>+</sup>.<sup>195</sup>

Clinical signs are not specific, but related with liver disease, such as anorexia, vomiting, diarrhoea, jaundice, abdominal pain or discomfort, pyrexia, fever and, in severe cases Disseminated Intravascular Coagulation (DIC) and hepatic encephalopathy, can be present.<sup>164</sup> Prognosis is poor, despite the therapy.<sup>164,195,196</sup> Primary splenic forms have been rarely reported. In this case, it is considered low-grade B type-marginal zone lymphoma-and animals tend to have a good prognosis, especially if asymptomatic at diagnosis and treated with splenectomy.<sup>197</sup>

#### 1.3.2.1.6 Ocular

Intra-ocular and peri-ocular lymphoma accounts for less than 0,5% of cases<sup>13</sup> and has been usually considered secondary to systemic disease<sup>198</sup> being described in 0,03 to 37% of cases with multicentric disease, usually associated with uveitis.<sup>199</sup> In intra-ocular lymphoma, peripheral T-cell lymphoma and DLBCL are the most common WHO subtypes and the percentage of primary cases, without systemic involvement has been described to be 61%, and associated with good prognosis without disease progression after enucleation.<sup>200</sup> Lymphoma affecting the conjunctiva tissue has a better prognosis than intra-ocular ones, since a great percentage of the latter progress to central neurologic disease.<sup>199</sup>

#### 1.3.2.1.7 Central nervous system lymphoma

Lymphoma affecting central nervous system is the third most common secondary tumour in brain and represents 1,8 to 4% of primary and 12% to 19,7% of secondary intracranial tumours with Rottweiler being the most overrepresented breed.<sup>201-204</sup> The most common presenting symptoms are seizures, ataxia, changes in mental status and paresis/paralysis.<sup>11</sup> Diagnosis can be made on basis of clinical history (underlying or previous lymphoma), Cerebrospinal Fluid (CSF) cytology, with or without Flow Cytometry (FC)<sup>168</sup> and diagnostic imaging, namely Magnetic Resonance Imaging (MRI).<sup>202</sup> Response to treatment is similar to some nodal forms as it is the prognosis.<sup>168</sup>

Post mortem diagnosis of brain intravascular lymphoma has also been reported. The majority of these cases are of T-cell immunophenotype and CD44<sup>+</sup>.<sup>205</sup>

#### 1.3.2.1.8 Other atypical and rare forms

Rare extra-nodal localizations of canine lymphoma have been reported, namely: renal,<sup>206,207</sup> bladder,<sup>208</sup> oral,<sup>209</sup> lingual,<sup>210</sup> nasal,<sup>211</sup> choanes,<sup>212</sup> prostatic,<sup>213</sup> vertebral,<sup>212,214</sup> muscular,<sup>215,216</sup> bone,<sup>217</sup> joints,<sup>218</sup> cardiac,<sup>219</sup> mammary gland,<sup>220</sup> peripheral nerves,<sup>221</sup> adrenal glands,<sup>222</sup> testicular<sup>223</sup> and uterine.<sup>224</sup>

#### *1.3.2.2 High and low grade lymphomas*

Based on clinical and biological features lymphomas can be further categorized in high-grade (aggressive) or low-grade (indolent).

High-grade lymphomas histologically comprise large cells with high mitotic rates and clinically are associated with poor response to therapy and short survival times.<sup>17,225,14</sup>

Diffuse Large B-cell lymphoma, Burkitt lymphoma, B and T-lymphoblastic lymphoma and Peripheral T-cell Lymphoma Not-Otherwise-Specified (PTCL-NOS) are the most common aggressive forms in dogs, with median overall survival times in treated animals varying according with the WHO entity, specific prognostic markers or the treatment used.<sup>14,18, 28, 183,226,227</sup>

The true incidence of canine low-grade or also designated Indolent Lymphomas (ciL) is uncertain<sup>228</sup> with variable total (5,3-29%) reported incidences.<sup>15,160,130</sup> The most common subtypes of ciL are T-zone Lymphoma (TZL), nodal(nMZL) and splenic Marginal Zone Lymphoma (sMZL), Follicular Lymphoma (FL) Mantle Cell Lymphoma (MCL), B Small Lymphocytic cell Lymphoma (B-SLL) and small clear cell/T-zone lymphoma; their incidences varies between studies.<sup>21,229</sup> T-zone lymphoma has a an active phenotype, presumably associated to antigen-driven mechanisms and is common in Golden Retrievers, a breed associated risk due to the presence of high levels of CD45<sup>+</sup> T-zone cells and T-cell receptor rearrangement, in healthy animals.<sup>230,231</sup>

Identification of pure extra nodal forms must relay on strict staging procedures and, if the disease has shown to be limited to a solitary site, a non-systemic therapy can be attempted such as surgery or local radiation therapy. In the presence of two or more extra nodal sites, it is recommended that should be assumed a systemic process and consequently, treating with systemic chemotherapy.<sup>11</sup> With an indolent clinical course, survival times are usually long and aggressive multidrug treatment only leads to partial responses.<sup>130</sup>

If in the past the therapeutic recommendations were close monitoring or "watchful waiting"; the actual evidence is that dogs suffering from indolent forms may experience aggressive disease courses.<sup>14,130</sup> Low-grade lymphomas, in general, usually progress slowly and only

show symptoms when the disease burden is big and are in advanced stages. This late symptomatic and aggressiveness is more common in T-cell types and, when detected, it is associated with poorer prognosis.<sup>14</sup>

The veterinary data on transformation of indolent lymphomas into more aggressive histological subtypes are rare; nevertheless, based on the existent reports, B-cell tumours such as FL, MZL and B-SLL, are more prone to transform than T-ZL.<sup>232</sup> In the particular case of nodal MZL, the only specific study published, found that all dogs presented in stage V, have histology with the diffuse pattern and loss of follicle-related architecture, designated by “late-stage” disease, in this cases, assuming the clinical behaviour of a high grade lymphoma.<sup>233</sup> In nodal MZL bone marrow infiltration significantly affects Lymphoma Specific Survival (LSS), the percentage of medullar invasion of less than 1% being associated with the longer median outcome (1403 days).<sup>234</sup>

#### **1.4. Diagnosis**

Several medical conditions can resemble lymphoma, and the list of differential diagnosis is long. From reactive adenopathy due to infectious diseases such as Leishmaniosis, Ehrlichiosis, Blastomycosis and Histoplasmosis, lymph node metastatic cancer, other haematological neoplasia (leukaemia, multiple myeloma and systemic histiocytosis) and immune mediated disorders (pemphigus, vasculitis, lupus erythematosus, polyarthritis, etc.).<sup>11</sup> Definitive diagnosis of a lymphoid malignancy should be based, for all patients, on a full clinical history, including epidemiological and travel history context, complete physical examination, serology, laboratory assessment of haematological and biochemical parameters, cytology and/or histopathology of the affected organs/tissues and complementary serology, imaging techniques such as X-rays (XR's) and ultrasound. More advanced laboratory techniques such as flow cytometry and clonality assessment may be necessary to confirm diagnostic and/or for staging purposes.<sup>11,16</sup>

##### *1.4.1 Clinical history and physical examination*

Previous diseases or immunizations, duration of clinical signs, concomitant diseases and possible exposure to risk factors and vector-borne agents should be assessed. Physical examination should include mucosal colour and aspect to assess the presence of icterus, ulceration or petechia. Lymphnode palpation should be based in a “head to tail” approach, including rectal digital assessment of sub-lumbar lymph nodes. Abdominal palpation should assess discomfort, hepatic and/or splenic enlargement and the presence of abdominal fluid.

Thoracic auscultation should be done carefully to discard the decrease/absence of respiratory sounds due to the presence of pleural fluid and/or mediastinal masses. Eyes and adnexal structures should also be assessed to detect abnormalities, such as uveitis or exophthalmos.

Dogs with high-grade lymphoma appear to have a relevant prevalence of intestinal opportunistic protozoa infection<sup>235</sup> and this should be taken in consideration when assessing clinical signs and laboratory data, since the presence of these infectious agents can lead to abnormalities in clinical signs (diarrhoea, cachexia, weight loss) and laboratory data (anaemia, hypoproteinemia) incorrectly associated with lymphoma.

### *1.4.2 Imaging*

When thoracic and abdominal X-rays are used for diagnosis and staging purposes, multiple abnormalities are more commonly seen than solitary changes, but thoracic radiography alone cannot be used for diagnosis or staging since up to 51% of the affected dogs can have normal chest XR's.<sup>236</sup> Most of the lymphoma radiographic features are non-specific, consisting of normal to alveolar and/or unstructured interstitial pulmonary infiltrates, nodules and/or masses and bronchial infiltrates, pleural effusion and lymphadenopathy.<sup>237</sup> Nevertheless, the most common finding is lymphadenopathy.<sup>194,238,239</sup> Radiographic findings on the shape and relationship between intra-thoracic structures can have a predictive value for differentiating between mediastinal lymphoma or thymic cancer, being the latter associated with a rightward shift of the cardiac silhouette on ventrodorsal or dorsoventral XR's views.<sup>240</sup> Pulmonary infiltration seems not to have a prognostic value, only if cranial mediastinal lymphadenopathy is present.<sup>241</sup>

On ultrasound, lymphoma affected lymph nodes appear mostly heterogeneous, with reduced hilar tissue definition.<sup>242</sup> Sonographic evaluation of the alimentary form of cL can give unspecific signs and in 26% of cases ultrasound features can be increased intestinal wall thickness and variable presence or absence of normal gut wall layering.<sup>243</sup> Spleen evaluation can demonstrate poorly defined hypoechoic to anechoic nodules and relative hypoechoic parenchyma, when compared with liver and kidneys.<sup>244</sup> Renal lymphoma ultrasonography findings consist normally of loss of in cortico-medullary distinction, renomegaly, renal deformity, hypoechoic lesion(s) and, rarely, hyperechoic lesion(s). Hypoechoic lesions can assume nodular shape or indistinct areas. In 30% of the cases ultrasound can reveal only minor abnormalities, including grade 1 pyelectasia, mild renomegaly, and focal loss of cortico-medullary definition. Bilateral lesions are normally seen in most of the cases.<sup>245</sup> Thoracic ultrasonography images can also be useful as a minimally invasive way to

differentiate between mediastinal lymphoma and thymic neoplasia, being the last most heterogeneous and with cavitory cysts.<sup>246</sup>

Computed Tomography (CT) to evaluate disease extension in cL is not commonly used. Nevertheless it seems that the majority of the cases with liver and splenic involvement will have no specific changes, other than associated lymphadenomegaly; normal hepatosplenic attenuation and enhancement is normal and no parenchymal nodules or masses are detected.<sup>247</sup> Contrast enhancement computed tomography assessment of renal lymphomas has being reported to show contrast homogeneous enhancement, common bilateral renal involvement and the presence of multiple masses without vessel enhancement detected in the cortico-medullary phase.<sup>248</sup> Computed tomography can also help to distinguish between lymphoma and thymus neoplasia, since the former on CT tends to envelop the cranial vena cava.<sup>249</sup>

Magnetic resonance imaging (MRI) is used manly when there are suspicious of disease affecting the central or peripheral nervous system, and on MRI lymphoma lesions have usually indistinct margins in T2-weighted caption, peri-lesional hyper intensity in Fluid-Attenuated-Inversion-Recovered (FLAIR) ponderation, associated with abnormal meninges around the tumour and only 50% of the lesions showing contrast enhancement.<sup>202</sup> Regarding spinal and para-spinal lesions, Short-Tau-Inversion-Recovery (STIR) and T1-W post contrast sequences, seem the most useful to identify lymphoma lesions.<sup>250</sup>

### *1.4.3 Clinical pathology*

#### *1.4.3.1 Haematology*

Complete haematology assessment, in dogs with lymphoma, can be completely normal or reveal moderate normochromic normocytic non-regenerative anaemia.<sup>251-253</sup> Blood loss from gastrointestinal bleeding, immune-mediated haemolysis,<sup>254</sup> bone marrow infiltration<sup>255</sup> and systemic inflammation<sup>256</sup> are causes that can contribute to decrease haematocrit.

Anaemia is a common finding in lymphoma patients being present in up to 47% of cases.<sup>257</sup> However, in dogs with stage V, anaemia can be present in 85% of patients.<sup>258</sup> In cases of primary renal involvement, polycytemia has been reported<sup>259</sup> and is associated with presumptive abnormal erythropoietin production.<sup>207</sup> Other than decreased cell count, erythrocyte morphologic abnormalities, such as acanthocytes, eccentrocytes and schistocytes, can be present.<sup>13</sup>

On the white cell department, chronic inflammatory leukogram, leukopenia and circulating blast percentages can account to 56,2%, 7,5% and 27,5%, of the expected alterations,

respectively.<sup>251</sup> Blood smear should always be assessed in order to characterize the cellular morphologic changes and identify the presence of circulating neoplastic lymphocytes. To further characterize circulating leukocyte populations, immunophenotype by flow cytometry can be performed, helping identifying the presence or absence of leukemic status and the possible original tumour (leukemic phase of a lymphoma vs. nodal involvement by a leukaemia).<sup>258,260</sup>

Hypereosinophilia as paraneoplastic syndrome associated with spinal lymphoma has also been described and lymphoma should be considered a cause when abnormal high eosinophil count appears.<sup>214</sup>

Regarding platelets, thrombocytopenia is the most common abnormality found, and its percentage varies widely in the literature, being found in 12,5%<sup>251</sup> to 36%<sup>261</sup> of patients. Also is more prevalent, 71% vs. 22%, and significantly more severe in T-cell than B-cell lymphomas, respectively.<sup>258</sup> The concomitant presence of thrombocytopenia and lymphocytosis is very suspicious of bone marrow infiltration by the lymphoma<sup>255</sup> suggesting further investigation. Secondary thrombocytosis can occur in 4,5% to 8,5% of dogs affected by lymphoma<sup>262,263</sup> and can be associated with mild haemostatic changes, mainly hypercoagulability present in 81% of cases at diagnosis. Thrombocytosis can persist until 4 weeks after completion of chemotherapy<sup>264</sup> or associated to on-going treatment with vincristine.<sup>265</sup>

When anaemia, thrombocytopenia, leucocytosis or lymphocytosis are present, bone marrow involvement should be considered, but cytopenias are not always predictive.<sup>255</sup> In the co-existence of two or more cytopenias, lymphoma-associated hemophagocytic syndrome should be considered and confirmed by the presence of more than 2% hemophagocytic macrophages in bone marrow aspirates and the concomitant presence of other clinical data: 1) fever, 2) splenomegaly, 3) cytopenias affecting two or three lineages, 4) hypertriglyceridemia, 5) hemophagocytosis in the bone marrow, spleen, or lymph nodes and 6) hyperferritinemia.<sup>266</sup>

The absence of peripheral blood abnormalities does not exclude bone marrow infiltration and despite not recommended by guidelines, a bone marrow evaluation should be included in the diagnosis and staging procedures.<sup>16,167,267</sup>

### 1.4.3.2 Serum Biochemistry

The presence and severity of serum component abnormalities is dependent of several factors, other than the lymphoma, namely: comorbidities, elapsed time since disease onset and diagnosis, affected organs and cell phenotype.

Total proteins can be elevated due to dehydration and/or clonal gammopathy ( $\beta$ -2 spike).<sup>268</sup> Hypoproteinemia secondary to subnutrition/malnutrition, mal absorption due to gastrointestinal tract infiltration<sup>173</sup> and bleeding episodes and protein loss can also lead to hypoalbuminemia.<sup>196</sup>

When liver is primarily or secondarily infiltrated, depending of its severity and extension, liver enzymes can be normal, slight or highly increased, decreased serum albumin and bilirubinemia can be also found.<sup>196</sup> Hypercalcemia is also a frequent finding, being present in about 67,5% of mediastinal forms of cL<sup>192</sup> and its frequent with T-cell phenotype,<sup>269</sup> namely CD4<sup>+</sup>.<sup>161</sup>

Increased values of nitrogenated compounds and creatinine are consequence of primary or secondary renal infiltration or associated conditions, such as dehydration caused by vomit and/or diarrhoea or nephrocalcinosis and levels of proteinuria are usually low.<sup>270</sup> Other biochemical alterations that can be present and reported in literature, are: low levels of hypocobalamine<sup>271</sup> higher levels of hydroxybutyrate and lactate,<sup>272</sup> low levels of vitamin D<sup>273</sup> positively mediated by levels of ionized calcium,<sup>274</sup> and high levels of alpha-fetoprotein, specially in stage V<sup>275</sup> and, increased levels of Lactate Dehydrogenase (LDH)<sup>276</sup> at diagnosis, probably due to increase in LDH2 and LDH3 isoforms.<sup>277</sup>

Serum proteomic profiling from dogs with lymphoma identified 19 protein peaks significantly different from the profile of controls. From these 19, two biomarkers had positive predictive value of 80% and negative predictive value of 88%, enabled to distinguish between lymphoma and non lymphoma affected animals.<sup>278</sup> Serum cytokines Monocyte Chemoattractant Protein-1 (MCP-1), Interleukin 6 (IL-6), and Interleukin 10 (IL-10) were shown to be significantly higher in dogs with lymphoma compared to healthy controls. In the same study, MCP-1 and IL-10 were significantly higher in the B cell lymphoma group than in the healthy controls and MCP-1 and IL-6 levels were significantly higher in the T-cell lymphoma group than in the healthy dogs.<sup>279</sup> To date there aren't biomarkers to successfully distinguish canine lymphomas by indolent or aggressive clinical behaviour or by immunophenotype.<sup>280</sup> The principal clinically accessible biomarkers are described in table 2.

**Table 2.** Commercially available serum protein biomarkers for cNHL and their association with phenotype, response to treatment and prognosis.

Biomarker	Comments	Clinical value
Endostatin	Higher in dogs with lymphoma compared with healthy controls. <sup>281</sup>	Not determined. <sup>281,282</sup>
MCP-1	Higher in dogs with lymphoma than in healthy dogs, correlates with stage and DFI <sup>283</sup>	Correlates with DFI using CHOP. <sup>283</sup>
Thymidine Kinase 1 (TK1)	Increased in dogs with lymphoma compared to healthy controls and dogs with non hematologic malignancies; B cell lymphoma have higher levels than T cell; TK levels don't correlate with stage disease; TK activity prior to treatment was not associated with DFR or survival. <sup>284</sup> In dogs with CR, an increase in TK1 activity measured at a 4-week interval predicted relapse. <sup>285</sup>	May correlate with prognosis and stage. <sup>284</sup> Can help predict complete responders and early progression. <sup>285</sup>
Vascular Endothelial Growth factor (VEGF)	VEGF was higher in high-grade compared with low-grade T-cell lymphomas. <sup>286</sup> Increased when compared with healthy controls. Significantly higher in T-cell lymphomas, and in stage V, when compared with stages III-IV, regardless of immunophenotype. <sup>286</sup> Circulating levels of VEGF significantly correlate with the WHO sub-stage b prognostic factor. <sup>287</sup>	VEGF at diagnosis may have an independent influence on DFI. <sup>287</sup>
Matrix Metalloprotein (MMP)	Affected dogs can have higher MMP-9; values significantly higher in T-cell lymphomas and in stage V compared with stages III-IV disease, regardless of immunophenotype. <sup>286</sup> Circulating levels of both MMP-2 and MMP-9 activities significantly correlate with the WHO sub-stage b <sup>287</sup>	No correlation was found between MMP's levels at presentation and outcome <sup>286</sup>
Haptoglobin	Increased in dogs with high-grade lymphoma, when compared with healthy controls. <sup>288</sup>	Possible indicator of remission/recurrence status. <sup>289</sup>
C-Reactive Protein (CRP)	CRP concentrations at diagnosis were above the reference range in 68% dogs with cL that returned to reference range in 90% of the dogs achieving complete. <sup>290</sup>	CRP may be useful in determining complete remission. <sup>290,289</sup>
Alpha-feto protein (AFP)	The concentration of AFP was high, especially on stage V lymphoma. Chemotherapy caused a decrease in AFP serum concentration, during both the induction and the maintenance phases of treatment, when compared to the same animals before therapy. <sup>275</sup>	Associated with stage V. <sup>275</sup>
Serum Amyloid A (SAA)	SAA concentration was significantly higher in dogs with lymphoma before chemotherapy compared with healthy and chemotherapy control dogs. No increase in SAA concentration was found at relapse. <sup>291</sup>	SAA cannot predict relapse in dogs with multicentric lymphoma. <sup>291</sup>
Alpha-1 acid glycoprotein (AGP)	Mean serum AGP concentration higher in dogs with lymphoma prior to treatment compared to healthy controls. Mean serum AGP concentrations 3 weeks prior to and at the time of relapse were significantly higher than concentration measured after the first dose of doxorubicin, and were not significantly different from concentration measured before treatment. <sup>292</sup>	AGP concentration may be a useful method of predicting relapse before recurrence of clinically detectable disease. <sup>292</sup>

**Legend:** MCP-1: Monocyte-chemotactic-protein-1; DFI: Disease-free-interval; CHOP: Cyclophosphamide-Hydraunorubicin-Oncovin-Prednisolone; TK1: Thymidine Kynase-1; DFR: Duration of First Remission; CR: complete Remission; VEGF: Vascular Endothelial Growth Factor; WHO: World Health Organization; MMP: Matrix Metalloprotein; CRP: C-Reactive Protein; AFP: Alfa Fetoprotein; SAA: Serum Amyloid A; AGP: Alpha-1 Acid Glycoprotein

#### 1.4.4 Cytology and histology

Once lymphoma is suspected, it is fundamental and necessary to perform a confirmatory morphologic test. This is commonly done in veterinary medicine by the use of Fine Needle Aspiration Cytology (cFNA) of the lesion(s).<sup>293</sup> This technique is easy to perform, low cost, minimally invasive and sensitive.<sup>294,295</sup> Despite its high sensitivity, this factor is highly dependent of the quality of smears and the experience of the examiner.<sup>296</sup> Other than solid structures like lymphnodes or organs, it can also be applied in the evaluation of the cellular

**Table 3:** Histological and cytological characteristics of the most common diagnosed entities of canine lymphoma. (Adapted)<sup>18, 44,130,160</sup>

WHO Subtype	IPT	Grade	%	Histology	Cytology
Diffuse large B cell	B	High	54	Diffuse, thinning of lymphnode capsule, compression of peripheral and medullary sinus, fading germinal centres, destruction of normal nodal structures, filling of the medullary cords with neoplastic cells, many tingible body macrophages, high mitotic rate.	Large cells, scant cytoplasm, uniformly large nuclei, usually round, rarely cleaved or indented, centroblastic and/or immunoblastic, mitotic figures common.
Peripheral T Cell -NOS	T	High	16	Diffuse, thin lymphnode capsule, paracortical expansion, sinus compressed, focally obliterated, spread of neoplastic cells to perinodal tissue. The neoplastic cells are usually large, may be of variable cell size, frequently have cleaved or oval nuclei, nucleoli inconsistent in number and size, variable mitotic rate, may lack numerous tangible body macrophages.	Large to variable cell size, frequently cleaved or oval nuclei, nucleoli inconsistent in number and size, pale cytoplasm, variable mitotic figures, few tingible body macrophages.
T zone lymphoma	T	Low	14	Diffuse paracortical expansion of the lymph node, uniform population of small or intermediate cells expanding in paracortex and medullary cords, no effacement of nodal architecture, The capsule is typically thin, nuclei no internal nuclear detail, shallow nuclear indentation, extended pale cytoplasm, mitotic rate variable. There are 2 subtypes: T-Zone lymphoma (TZL) of small cells, with nuclei only slightly larger than red cells, and TZL of intermediate-sized cells. Mitoses are not present in most fields.	Small cells, round nucleus, can have irregular shallow nuclear indentation, extended pale cytoplasm (hand mirror shape).
T lymphoblastic	T	High	5	Thin capsule, focal perinodal colonization, diffuse cortical and medullary filling, moderate anisokaryosis, nuclei from round to oval or irregularly indented, densely stained cells (dispersed chromatin) that obscures nucleoli, no tingible body macrophages, high mitotic rate.	Uniform population intermediate-sized cells, evenly dispersed chromatin, obscured nucleoli.
Marginal zone	B	Low	4	Coalescing aggregates of indolent B-cells surround fading remnants of germinal centres, resembling the marginal zone of a lymph node follicle.	Intermediate-sized nuclei, prominent single central nucleoli, abundant lightly stained cytoplasm, no mitotic figures.

**Legend:** WHO: World Health Organisation; IPT : Immunophenotype; %: percentage; NOS: Not-Otherwise-Specified

content of thoracic or abdominal fluids.<sup>297</sup> Cytology can be achieved without major restraint; it can be done without anaesthesia or sedation, reducing drastically the costs and when there is enough aspirated material further diagnostic techniques such as cell block cytology,<sup>298</sup> clonality,<sup>299</sup> flow cytometry,<sup>300</sup> and Immunocytochemistry (ICC)<sup>294</sup> can be performed. Although cytological exam can be of limited diagnostic value in the presence of hyperplastic reactive lymph nodes,<sup>11</sup> in small cell and follicular lymphomas,<sup>44</sup> it can give accurate diagnosis, when additional immunophenotyping is used.<sup>144</sup> Gastrointestinal samples obtained by endoscopic cytology can also be used for diagnostic purposes with a concordance with histopathology of 81,4%, a sensitivity of 98,6%, a specificity of 73,5%, a positive predictive value of 72,3%, and a negative predictive value of 98,6%.<sup>301</sup>

Classification according the WHO needs to be done on tissue obtained from an incisional biopsy, a tissue biopsy or a total lymphadenectomy, to assess tissue architecture, growth pattern, nuclear characteristics, mitotic index, nuclear size and immunophenotype. The cytological and histologic characteristics of the most common canine lymphoma entities are described in Table 3.

For histology it is preferable to remove all lymph node including capsule, in this way the pathologist can assess all node morphology and identify normal architecture disruption and invasion of capsule and surrounding tissues.<sup>15</sup>

The studies done so far demonstrated that most categories of cL are aggressive high-grade tumours,<sup>144</sup> DLBCL being the most common subtype.<sup>15,302</sup> Despite the fact that tumour biopsy needs anaesthesia, is more invasive and increases the costs, it should be standard practice since it helps in the diagnosis of low-grade lymphomas and allows classification in subtypes, consequently, associating the entity with respective clinical behaviour and prognosis.<sup>14</sup>

#### *1.4.5 Diagnostic Accessory tests*

##### *1.4.5.1 Immunophenotyping*

Proteins synthesis is the end product of a cascade that starts at gene level. The possibility to detect specific cellular proteins in cytological or histologic samples was a great scientific achievement and contributed strongly to help understanding tumour biology and reaching a reliable diagnosis.<sup>303</sup>

To the author's best knowledge, the first description of immunophenotyping for B and T-cell origin in formalin fixed paraffin embed samples from dogs, was done in 1984 using murine derived monoclonal antibodies, that identified lymphomas as 78% of B-cell origin, 10% T-cell and the lasting remained non identifiable.<sup>304</sup> Antibodies can be applied in frozen or formalin

fixed paraffin embedded sections of lymphoma,<sup>149</sup> free cells presented in a smear,<sup>305</sup> or in a cell suspension.<sup>306</sup> The most common used epitopes for immunophenotype determination in canine lymphoma are, for B-cells: CD79<sup>a</sup>, CD20, CD21 and PAX-5, and for T-cells: CD3, CD4, and CD8.<sup>15</sup>

The use of multiple epitopes for the same lineage should be considered since as, previously reported, the use of a single T or B-cell marker cannot be able to accurately identify cell-lineage, especially in B-cell tumours.<sup>307</sup> We demonstrated that not a single antibody could detect all B-cell lymphomas and, in some cases, there was cross reactivity between B and T-cell markers.<sup>308</sup> In cases were cells miss the expression of common T and B markers, designated by “null cells”, the presence of an Anaplastic Large cell lymphoma must be suspected, and its diagnosis can be confirmed by CD30 positivity.<sup>309</sup> When cells loose the expected immunophenotype labelling and have aberrant expression of CD molecules, which can be common in cL, it should be considered as valuable criteria for high malignancy.<sup>170,310,311</sup> Despite its great value in routine diagnostic, immunophenotyping should not rely only in Immunohistochemistry (IHC) and more sensitive techniques, such as Polymerase Chain Reaction (PCR) for Peripheral Antigen Receptor Rearrangement (PAAR), should be performed concurrently to increase sensitivity in the diagnosis of lymphoma.<sup>312</sup> Also, cell blocks method from fine needle aspiration of malignant lymph nodes, despite failing to help complete immunophenotyping of T-cell lymphomas, missing more than 83% of the cases, had a good performance for B-cell tumours, where it was possible to identify 96% of the samples.<sup>298</sup>

Besides B or T-cell classification, IHC can be used to distinguish cellular maturation status,<sup>313</sup> prognostic subtypes,<sup>183,130</sup> assess proliferation ratios,<sup>314,315</sup> angiogenesis,<sup>316-318</sup> prognostic markers such as p-glycoprotein,<sup>318</sup> double c-myc/Bcl2 expression,<sup>225,319</sup> survivin<sup>316</sup> and even therapeutic targets as transferrin,<sup>318</sup> folate receptors,<sup>318</sup> retinoid receptors,<sup>318</sup> tyrosine kinase receptors,<sup>320</sup> or topoisomerase II.<sup>321</sup>

Recently, immunophenotyping characterization of splenic follicular derived MZL and MCL lymphomas was described: both canine MZL and MCL are both positive for B-cell markers CD20 and CD79<sup>a</sup> and negative for the T-cell marker CD3. Expression of CD5 is frequent in MZL. Following identification of B vs. T -ells with CD20 and CD3, a panel composed of Bcl2, Bcl-6, MUM-1 and MCL-1, combined with the histological and morphological pattern can be used to accurately differentiate MZL and MCL in dogs, with constant expression of Bcl2, inferior expression of MUM-1 and lack of MCL-1 expression in MCL.<sup>322</sup>

In human medicine, important IHC algorithms are applied to DLBCL to identify prognostic groups: Hans algorithm, one of the most basics, uses the different immune labelling for CD10, BCL-6 and MUM-1 to categorize hDLBCL as Germinal Centre B-cell like (GCB) and non-

germinal centre or activated B-cell like (nGCB), anticipating treatment response and prognosis.<sup>323</sup> We have analysed and described the application of Han's algorithm, using a tissue micro-array to a case series of cDLBCL, but the rare expression of Bcl6 and MUM-1 in the tumours contributed to the reported insuccess.<sup>324</sup> In our review, presented at the European Canine Lymphoma Network meeting in Lugano, the difficulties in having available antibodies that cross-react with the canine species for the specific epitopes used in the algorithms described for hDLBCL is the major limitation for the evaluation of immunostain based diagnostic algorithms in cDLBCL.<sup>308</sup> Further attempts were made using IHC to apply Han's algorithm to cDLBCL, but our previous results were confirmed, that by IHC, Bcl6 and MUM-1 expression is rare, and only by gene expression analysis seems to be effective to differentiate canine GCB and nGCB DLBCL, based on signalling and molecular signatures, similar to GCB and non-GCB in humans.<sup>83,325</sup>

#### *1.4.5.2 Flow cytometry*

Flow cytometry is a very useful technology, developed in the last 30 years that enables to study cells suspension in liquids. It has several applications in the areas of immunology, molecular biology, cancer biology and infectious diseases monitoring. The characterization of normal canine lymphnode lymphocyte subpopulations by flow cytometry was made by Rutg en and co-workers (2015).<sup>326</sup>

Flow cytometry has been of great applicability in immunophenotyping lymphomas, and can be of more precise value in specific T-cell types, such in T-zone lymphoma were it is routinely diagnosed by this technique based on an expansion of small to intermediate sized CD3+CD5+ T-cells with characteristic loss of the pan-leukocyte marker CD45.<sup>107</sup> When CD45 negative expression exists, a small clear cell/T-zone distinct lymphoma subtype that shows an indolent clinical course may be presumed in dogs.<sup>229</sup> The negative expression of CD45, either on T or B lymphoma cells, is attributed to phenotypic variant from the CD45<sup>+</sup> cells.<sup>327</sup>

The majority of nodal PTCL cases (82%) are characterized by CD4 expression by flow cytometry and fewer cases (6,8%) express CD8 and are negative for CD4 and CD8 (11,0%)<sup>107</sup>. Some cases can also co-express CD20/CD3 and BLA36/CD3 molecules, as previously documented.<sup>308</sup>

Also, a subset of TZLs co-express CD21, a B-cell marker, and CD3 being this bilineage possibly related to a specific subtype of T cell lymphoma, more than an atypical dual phenotype.<sup>325</sup>

#### *1.4.5.3 Polymerase Chain Reaction and cell clonality detection*

The synthesis of immunoglobulin chains in B cells and T-cell receptors in T cells, comprises a number of processes at the molecular level, involving gene deletion and recombination, to produce a great variety of antibodies against all the possible pathogens. During maturation, lymphocytes acquire unique antigen receptors through rearrangements of VDJ regions in T and B cell receptor genes (T-cell receptor gamma gene (TCR $\gamma$ ) and Immunoglobulin Heavy Chain gene (IGH), respectively). A clonality assay is a laboratory procedure done to determine the presence of a clonal receptor locus, derived from the same cell or small group of cells. Assuming that once antigenic stimuli disappears, all the benign clonal expansions reacting to an antigen will be reduced to a small number of memory cells, the presence of a persistent and growing clonal population, can be suspicious to belong to a neoplastic condition. PARR has been applied in research and clinical fields to distinguish between neoplastic (clonal or oligoclonal) and reactive (polyclonal) lymphoid proliferations. This technique was first described, in veterinary oncology, by Vernau and colleagues (1999)<sup>328</sup> for canine T cell immunoglobulin gene receptors, and lately validated by Burnett and colleagues (2003)<sup>329</sup> describing the primers for the most common rearrangement combinations and PCR conditions to obtain either B and T cell clonally assessment in canine lymphoma. Over the years several variations of the primary technique have been described. Decoding of canine genome led to increase gene coverage<sup>330,331</sup> and technique optimizations.<sup>299,332-335</sup> In the particular case of TCR's the identification of more gene rearrangements<sup>336</sup> and consequently the availability of more primers, increased the detection rate up to 100%.<sup>337</sup> The PARR assay can be done using either formalin fixed tissues, blood or stained/unstained air dried smears and has an estimated sensitivity between 72-100% and specificity of 96-100%, depending of the primers used and technique.<sup>299</sup> Nevertheless infections with *Ehrlichia canis*<sup>329</sup> and *Leishmania infantum* can lead to clonal peaks leading to a false-positive test. In the specific case of *Leishmania* infection, in a recent study,<sup>296</sup> 9,7% (3/31) of cases showed a monoclonal arrangement; however, a polyclonal background was always present and should be taken into consideration, along with other clinical signs and tests. The true value and sensibility of PARR to detect gastrointestinal lymphoma is still low, with the best reported sensibility being 76%; nevertheless in the same study, almost half of the dogs with chronic enteritis had also clonal lymphocyte populations.<sup>338</sup> The right choice of primer sets to identify clonality in canine T cell lymphoma is fundamental to achieve high sensitivities, being the primers designed by Keller and co-workers (2012)<sup>337</sup> the ones demonstrating the highest detection rates<sup>339</sup> Despite being a widely available technique and commonly used for diagnosing and clinical staging of cL it should be combined with other techniques, namely flow cytometry, in

order to improve its clinical usefulness. PARR usefulness for phenotyping lymphomas should only be used when there are no samples available for IHC or FC.<sup>340</sup> Recently, its use to diagnose renal lymphoma in dogs was also described, through the detection of lymphocyte clonal populations in urine.<sup>206</sup>

Clonality assessment can also help to differentiate mediastinal lymphoma from thymoma, since the first is mainly clonal, while the majority of thymomas have an infiltration of polyclonal populations of lymphocytes.<sup>341</sup> Immunoglobulin chains or TCR's are not the only way to assess clonal expansion in hematopoietic diseases; it has been described another technique based on X-chromosome inactivation pattern analysis (XCIP), where the authors could identify clonal patterns in haematological malignancies in female dogs.<sup>342</sup>

### 1.5. Evaluation of tumour burden: Staging

Staging is crucial for the clinical management of lymphoma, to substantiate treatment decision and to elaborate prognosis. Throughout staging procedures, it is possible to assess the distribution and extent of the tumour, either at diagnosis or follow-up, evaluating response to treatment and lately attributing precise stratification of patients to potentiate accuracy in clinical research. Additionally, knowing the sites of involvement at diagnosis makes it possible to accurately restage at the end of therapy and confirm a complete remission.<sup>343</sup> Unfortunately, not every owner accepts to go forward with full staging procedures, frequently because of financial restraints or worries about the pain associated and invasiveness of certain procedures.<sup>344</sup>

**Table 4:** Lymphoma staging system (Owen, L.N., 1980)<sup>345</sup>

<b>Stage I</b>	Involvement limited to a single node or lymphoid tissue in a single organ
<b>Stage II</b>	Involvement of many lymph nodes in a regional area ( $\pm$ tonsils)
<b>Stage III</b>	Generalized lymph node involvement
<b>Stage IV</b>	Liver and/or spleen involvement (2 Stage 111)
<b>Stage V</b>	Manifestation in the blood and involvement of bone marrow and/or other organ systems ( $\pm$ Stages I-IV)
<b>Substage</b>	
a) Without systemic signs	
b) With systemic signs	

The result of staging procedures and allocation of the patient with lymphoma to a WHO stage, significantly affects treatment decisions and is a major determinant in prognostic.<sup>346,347</sup> An accurate and reproducible staging methodology is, then, mandatory. The WHO staging scheme for cL<sup>345</sup> is summarized in Table 4. This scheme is largely applied to cL but its true

application to extra-nodal forms (MALT, CNS, Cutaneous) or splenic marginal zone lymphoma is very limited.<sup>343</sup>

Stage is attributed having in consideration several informations: clinical history to attribute sub-stages (a vs. b), physical examination, imaging techniques and peripheral blood/bone marrow assessment. The addition of several or more sensitive staging techniques, namely specific laboratory tests such as PARR,<sup>348</sup> flow cytometry<sup>260</sup> and imaging techniques such as Positron Emission Tomography (PET)<sup>349</sup> can increase the accuracy of staging, although it is not related with a more accurate determination of prognosis.<sup>350</sup> In a review study, where the author participated, made by the European Canine Lymphoma Network (EU-cLN) to assess staging methods described in published literature for canine nodal lymphoma, it was concluded that staging methods were inconsistent among studies. The most commonly performed staging procedures and their relative percentage on the literature in the last 15 years were: Complete Blood Cell count (CBC), serum biochemical profile (83,1%) and/or urinalysis (100%), thoracic radiographs (82,7%), abdominal radiographs (28,8%), abdominal ultrasound examination (59,6%) and bone marrow evaluation (73,1%).<sup>16</sup> A consensus guidelines was elaborated and recommends as staging procedures for cL: chest radiographs, abdominal ultrasound, histology/cytology and advanced imaging (CTscan/MRI) in cases of central involvement suspicion.<sup>351</sup> Nowadays, bone marrow sampling is not mandatory by official guidelines<sup>167,351</sup> because its clinical relevance and prognostic value stills remains controverse,<sup>255,343</sup> nevertheless, the EU-cLN, keeps recommending it.<sup>16</sup>

Lymph node size assessment, comparing the difference in size from pre-treatment to after treatment, is the basis for an evaluation to response to therapy. Due to the wide variation in companion animal breed size, the minimum measurable lesion/node in dogs as been set at 10 mm and for the purposes of assessing response, at least one target lesion must measure  $\geq 20$  mm at pre-treatment baseline for reliable assessment of response and progression.<sup>167</sup> Due to recent progresses, and if only staging is made according with these guidelines, that do not consider microscopic disease, it can now be considered very likely to overstate complete remission rates and understate progression rates.<sup>16</sup>

## **1.6. Treatment**

Lymphoma in dogs, as in humans, is a very heterogeneous disease comprising different histotypes, clinical and anatomical presentations. The therapeutic approaches are, as well, multiple. The vast majority of reported literature uses cytotoxic drugs on multicentric intermediate to high-grade lymphomas, being limited the available information on treatment for the indolent and low grade forms of cL.<sup>13</sup> Also, the vast majority of published literature in

treatment evaluation are case series, where there is a lack of controls and increased levels of standards of evidence-based medicine.<sup>189</sup> Without adequate treatment it is estimated that life expectancy for dogs with intermediate to high grade lymphomas is around 4 to 6 weeks.<sup>11</sup> Multiagent chemotherapy protocols are the therapy of choice for intermediate to high-grade lymphomas, while other therapeutic approaches (radiation therapy, surgery) can be used for local or low-grade tumours. Veterinary treatment choice and implementation are strongly determined by clients and, in oncologic patients the possible side effects and complications, associated with costs and comparative shorter survival times of dogs, can determine the client perspective on treatment choice and compliance. Determinant factors for case management success such as clinician experience with oncologic cases or the decision to referral for specialist care, are strongly dependent of practitioner perception of patient health status, the interaction between the client's bond with the dog and the client's financial status, practitioner experience with treating cancer, how worthwhile practitioners considered treatment to be and confidence in the referral centre. This subjective decisions impact patient survival and quality of life, with only 53,6% of the veterinarians in Ontario considering to refer a case of cL or osteosarcoma,<sup>352</sup> not always having in mind the objective best interest for the animal. In the United States of America, a survey to veterinarians attending a specialist cancer conference concluded that staging and treatment recommendations for canine lymphoma are multiple and about 31% of veterinarians treat B- and T-cell lymphomas differently.<sup>344</sup>

### *1.6.1 Pharmacologic resistance in canine lymphoma*

Intrinsic or acquired Multidrug Resistance (MDR) by tumour cells comprise a complex, not completely understood process, and remains a major obstacle to the long-term success of cancer chemotherapy.<sup>353</sup> Currently, 90% of chemotherapy therapeutic failures are during the invasion and metastasis of cancers related to drug resistance caused by several underlying mechanisms, comprising: 1) increased efflux of the drug, 2) inactivation of the drug, 3) multi-drug resistance, 4) evasion to apoptosis, 5) changes in drug metabolism, 6) epigenetic and drug targets and 7) enhance DNA repair and gene amplification.<sup>353,354</sup>

The role of Cancer Stem Cells (CSC) in drug resistance and lymphoma relapse and progression should be strongly considered, especially its contribution to tumour repopulation with treatment resistant cells. Cytotoxic treatment contributes to CSC selection, and cL dogs with B-cell neoplasms have subpopulations of CSC with the immunophenotype CD34<sup>+</sup>, CD90<sup>+</sup>, CD117<sup>+</sup>, and Oct3/4<sup>+</sup>, on the other hand, in T-cell lymphomas, the most prominent CSC subpopulation have an Oct3/4<sup>+</sup> immunophenotype, despite the presence of

some overlap between normal and malignant B and T-cells with respect to CSC marker expression.<sup>355</sup>

In the specific case of cL, relatively little is known about the underlying mechanisms of drug resistance. In line with the situation in human oncology, most studies have focused on the role of ABC-transporters and P-glycoprotein (P-gp) in particular, both *in vitro* and *ex vivo* in cell cultures and tissue samples, respectively. P-gp can be expressed in more than half of the canine lymphomas, with overexpression in high grade T cell immunophenotype.<sup>356</sup> Due to its importance to develop either severe chemotherapy reactions and disease progression,<sup>357</sup> when indicated (breed, co-morbidities, treatment choice) it can be necessary to know the baseline status of the patient for MDR1 before treatment.

Zandliev and colleagues (2015) demonstrated that intrinsic MDR was more common in T-cell than in B-cell lymphoma. Despite ABC-transporter, mRNA expression is common in cL, it is unlikely to be the sole cause for drug resistance. When assessing differences between the two cell types, drug resistance in TCL was associated to overexpression of ABCB1 and in B-cell drug resistance was associated with increased ABCG2 expression.<sup>111</sup>

### 1.6.2 Chemotherapy

A wide range of different single agent or multidrug combination protocols has been published in literature in the last three decades. The main therapeutic goals in veterinary oncology are: to achieve a high or complete response rate, long remission periods and have controlled and adequate toxicity with ratio cost/results acceptable.

Monodrug therapy protocols other than doxorubicin don't tend to result in durable clinical response;<sup>358,359</sup> in the specific case of cytosine arabinoside,<sup>360</sup> ifosfamide,<sup>361</sup> bleomycin<sup>362</sup> and gemcitabine<sup>363</sup> very low efficacy to treat cL is observed.

Prednisone as unique therapy, has low response rate and short duration of remission,<sup>364</sup> being the level of response mediated by the number of glucocorticoid (GC) receptors present in the neoplastic cells.<sup>364</sup> Its therapeutic effect, when included in a multidrug protocol can be discussable.<sup>365,366</sup> Despite its unclear evidence in treatment of cL, the rationale for carry on using glucocorticoids in the treatment of lymphoid tumours can be explained by the effects of GC on the immune system: 1) induction of lymphoid cell apoptosis (activation of the intrinsic pathway), 2) cell-cycle arrest and 3) inhibition of inflammation (repression of pro-inflammatory transcription factors including NF- $\kappa$ B and Activator Protein-1)<sup>367</sup>. Resistance to prednisolone develops quickly and in all lymphoma patients treated with glucocorticoids, the underlying mechanism is still not understood but decreased expression of the glucocorticoid receptor (NR3C1 $\alpha$ ) could play a role in conferring GC resistance<sup>368</sup>

Remission rates and duration on single agent therapy are short when compared to multi-drug combination therapy. To the date, the addition of several other drugs with known activity against malignant lymphocytes, have not increased significantly response rate, duration or remission or survival time than the obtained with standard Cyclophosphamide-Hydroxydaunorubicin-Vincristine (Oncovin®) and Prednisolone (CHOP) protocol, and sometimes adding only additional toxicity<sup>369</sup>. Chemotherapy maintenance, after remission has not proved to be superior over short maintenance free protocols, since protocols with no maintenance phase have comparable remission periods and progression free survival rates and appear to easier induce second remissions than their counterparts receiving maintenance therapy.<sup>11,370,371</sup> If the patient has received the doxorubicin cumulative dose of 180mg/m<sup>2</sup>, Mitoxantrone can be used to substitute doxorubicin with similar results in outcome and tolerance.<sup>372</sup> The same cannot be applied to the substitution of Vincristine, generally associated with gastrointestinal toxicity, by Vinblastine, since at usual dose has limited effect on malignant lymphoma.<sup>373</sup>

An overview of some of the published chemotherapy protocols with comments on their heterogeneity in study design and end points evaluation is provided in the table 5.

On a recent systematic review where the author participated, CHOP or CHOP-based protocols were the most common used first-line treatment in canine lymphoma accounting up to 71,4% of the used protocols reported in literature.<sup>16</sup> When used, CHOP protocol induced a Response Rate (RR) of 80-95% with median survival times between 10-12 months, being these reported values dependent of the presence of associated prognosis factors, which can affect response and survival rates.

In this systematic review the authors identified several pitfalls in first-line therapy studies, such as: 1) relative low number of patients in the treatment and/or control groups; 2) in 41 (65,1%) studies, treatment response analysis was based on subjective measurement of peripheral lymph nodes; 3) in none of them, confirmatory nodal cytology was described as mandatory to evaluate remission status; 4) methodologies were not described in 11 (17,5%) of the studies; 5) study endpoints such remission duration and survival were described only in 57 (90,5%) and 52 (82,5%), respectively; 6) the vast majority of studies have been compromised by the co-presence of a variety of lymphoma subtypes; 7) different WHO stages and 8) different inclusion criteria in the analysis of outcome, making it hard to assess the true response rate and clinical effectiveness of any given protocol or prognostic factors.<sup>16</sup> This inconsistent data compromise somehow the studies scientific value, having in account that canine non-hodgkin lymphomas includes different types of tumours, with different biologic behaviours, patterns of chemotherapy sensitivity and treatment responses, like in Humans.

**Table 5:** Comparison of the most commonly used chemotherapy protocols as first line treatment for high-grade multicentric canine lymphoma.

Protocol Designation	Dogs (n)	RR (%)	DFI (Days)	OST (Days)	1 year CRR(%)	2 year CRR(%)	1 year OST(%)	2 year OST(%)	IPT/ Subtype	References
COP	77	75	180	225	19	NR	19	NR	Mix/ NR	11,374
L-CHOP 12 wks	77	93 <sup>e</sup> 76	<sup>f</sup> 243	NR	28	16	NR	NR	Mix/ NR	375
CHOP 15 wks	134	98	177	311	NR	NR	NR	NR	Mix/ NR	347
<sup>d</sup> CHOP 19 wks	19	94,8 <sup>e</sup> 89,5	245	351	16	NR	16	NR	Mix/ <sup>g</sup> Y	376
L-CHOP	51	94	282	<sup>d</sup> 397	NR	NR	NR	NR	NR/ NR	12
<sup>a</sup> D+P	33	83	147	182	NR	NR	23	NR	B/ NR	358
LOPP	31	97 <sup>e</sup> 90	176	323	NR	NR	<sup>i</sup> 39	<sup>i</sup> 25	T/ NR	377
D	18	78	169,5		11	11	11	11	B/ Y	378
RA	54	98	<sup>c</sup> 194	NR	NR	NR	NR	NR	Mix/ NR	379
COPLA/LVP	75	92	175	252	17	5	NR	NR	NR/ NR	379
<sup>m</sup> L+P	36	78	106	NR	NR	NR	NR	NR	T/Y	380
<sup>n</sup> L+P	17	53	39,5	112	NR	NR	NR	NR	NR/NR	381
<sup>k</sup> C+P	20	64,3	NR	<sup>l</sup> 535	NR	NR	55	NR	T/ NR	176
<sup>l</sup> ImunCHOP	12	71,4	<sup>h</sup> 304	505	16,7	NR	NR	NR	B/ Y	382
Tel-eVax/ CHOP	17	NR	NR	452	NR	NR	NR	NR	B/ Y	383
dTERT-COP	14	NR	NR	684	NR	NR	NR	NR	B/ N	384
VELCAP-S	82	68%	140	NR	NR	NR	NR	NR	NR/ NR	369
CEOP	36	97	<sup>c</sup> 100	155	2,7	2,7	2,7	2,7	T/NR	385
LEOP	24	97	<sup>c</sup> 269	327	8	8	8	8	T/NR	
Pred	49	41	<sup>i</sup> 53	NR	NR	NR	NR	NR	NR/ NR	364

**Legend:** N: number of patients; RR: Response Rate; %:percentage; DFI: Disease Free Interval; OST: Overall Survival Time; CRR: Clinical Response Rate; IPT: Immunophenotype; WKS: Weeks; <sup>a</sup> 64% received L-asp; <sup>b</sup> With COP rescue; <sup>c</sup> Progression Free Interval; <sup>d</sup> With multiple rescue protocols; <sup>e</sup> Complete response; <sup>f</sup> For complete responders; <sup>g</sup> Indolent vs. High grade; <sup>h</sup> Time To Progression; <sup>i</sup> Rescue therapy; <sup>j</sup> Duration of remission ( not specified); <sup>k</sup> Only in 15/20 dogs, low-grade gastrointestinal lymphoma; <sup>l</sup> Dogs achieving complete response and partial response ;<sup>m</sup> Cutaneous form; <sup>n</sup> Multicentric form

When the tumour relapses or stops to respond to a specific combination of drugs in a protocol, a designated rescue protocol is offered.<sup>11</sup>

Several aspects are usually taken into consideration when choosing the rescue protocol; such as if it is the first relapse after full remission with induction therapy, the cumulative received doxorubicin doses, owner compliance, clinical score of the patient and clinician's preferences. When the relapse occurs during first-line therapy is assumed that tumour cells have acquired resistance and the use of new drugs (not yet administered) is recommended. If the relapse occurs after completion of first line therapy, a rescue protocol can be the same as the used for induction. Whichever the rescue protocol used, the response is always less effective and with some protocols toxicity can be higher.<sup>386-390</sup>

The majority of the studies on rescue protocols (Table 6) have most of the same pitfalls that first line protocols studies, making it difficult to infer on the true value of chemotherapy protocols for relapsed cL.

**Table 6:** Most commonly used rescue protocols for canine lymphoma.

Protocol Designation	Dogs (n)	RR (%)	MDR (Days)	IPT/ Subtype	References
<sup>a</sup> Lomustine	43	27%	90	NR/ NR	390
Mitoxantrone	15	47%	90	Mix/ NR	391
DMAC	100	35%	<sup>b</sup> 151	Mix/NR	389
DMAC	54	72%	<sup>c</sup> 112	Mix/NR	392
LOPP	44	<sup>d</sup> 27	<sup>c</sup> 112	NR/ NR	388
BOPP	14	<sup>d</sup> 28,5	<sup>c</sup> 129,5	NR/ NR	388
Bleo/Cytarabine	19	36,8	<sup>e</sup> 15	Mix/ NR	393
LPP	41	61	<sup>b,c</sup> 84	Mix/ NR	394
Tanovea®	50	77	172	B/ NR	395

Legend: <sup>a</sup> Different induction protocols; <sup>b</sup> TTD -Time to discontinuation; <sup>c</sup> only dogs with complete remission (CR); <sup>d</sup> CR; <sup>e</sup> Time-To-Progression; RR: Response Rate; MDR: Median Duration of Response; IPT: Immunophenotype; Bleo: Bleomycin; DMAC: Dexamethasone-Melphalan-Actinomycin-D and Cytosine Arabinoside; LOPP: Lomustine-Vincristine-Procarbazine and Prednisolone; BOPP: Carmustine-Vincristine-Procarbazine and Prednisolone; LPP: Lomustine-Procarbazine-Prednisolone

While true cures are rare, chemotherapy typically provides remission, improved quality of life and minimal toxicity<sup>396</sup> in veterinary medicine and doesn't seem to affect significantly dog's quality of life, even in very old animals<sup>397</sup>; nevertheless, side effects and complications can occur. In the two published questionnaires evaluating owners perspective of quality of life (QoL) of dogs receiving chemotherapy for their lymphoma, 53,3% of owners realized that QoL improved<sup>398</sup> and 68% acknowledge that was the same as before lymphoma during chemotherapy.<sup>399</sup> Notwithstanding treatment side effects, 65% of the owners were satisfied by the chemotherapy results in their dogs.<sup>398</sup> Thirty-two per cent felt that their pet's quality of life on chemotherapy treatment was acceptable but poorer than before the lymphoma occurrence.<sup>399</sup> Ninety-two per cent of owners had no regrets about treating their dog with multidrug chemotherapy and while many dogs developed treatment complications during multidrug chemotherapy, most owners felt that the quality of life of their pets was good during treatment and had no regrets about opting for palliative chemotherapy.<sup>399</sup> Treatment also doesn't seem to affect particularly immune-status, although with treatment initiation the neutrophils oxidative burst decreases over time, whereas phagocytic capacity improves comparing with baseline and to healthy controls.<sup>123</sup> Also, chemotherapy doesn't seem to compromise immunity from previous vaccinations to Canine Distemper, Canine Parvovirus

(CPV) and Rabies virus.<sup>124</sup> We previously demonstrated that after the administration of two cycles of CHOP the previously acquired immunity through vaccination or natural exposure to CPV does not appear to be significantly affected and CPV location and load profile in treated animals could be considered similar to that of healthy animals.<sup>400</sup> Nevertheless, dogs with lymphoma show signals of innate immune dysfunction and can be more prone to develop microbial infections, even in the presence of normal circulating white blood cells. This susceptibility is due to cytokine imbalance in pro- and anti-inflammatory cytokine production, namely diminished Tumour Necrosis Factor (TNF) production, that does not improve with remission status.<sup>401</sup>

A way to decrease toxicity and increase the therapeutic success of the cytotoxic molecules is to administer it encapsulated on liposomes. In veterinary medicine liposomal chemotherapy still has not got acceptance for use in a particular cancer.<sup>402</sup> When used in dogs affected by lymphoma, liposomal pegylated-doxorubicin didn't show any significant advantage<sup>403</sup> in clinical response and further tests are needed to support its use.

### *1.6.3 Surgery*

Surgery is not often used as a treatment for non-hodgkin lymphoma in dogs, mainly because of the multicentric and high-grade nature of the disease. Nevertheless, it may have some important roles in stage I disease, for diagnostic purposes (biopsy, lymphadenectomy), elective surgery or in a palliative setting, to remove obstructing nodes. In specific subtypes such as splenic marginal zone lymphoma<sup>197</sup> and follicular colorectal lymphoma<sup>178</sup> the curative role of surgery is described. On the palliative setting, surgical resolution of acute abdomen due to gastrointestinal lymphoma obstruction/rupture, or enucleation due to ocular lymphoma with organ destruction, can also be considered. In the particular case of intestinal lymphomas, surgery cannot guarantee total removal, since there is a high possibility that lymphoma infiltrates and spreads far beyond the macroscopic intestinal mass.<sup>404</sup>

### *1.6.4 Radiotherapy*

Due to the commonly systemic presentation of canine lymphoma, radiation therapy has restricted application. It can be used to treat local disease, with curative intent in particular cases of stage I, extra nodal forms or in the palliative setting. Low-dose total body irradiation (TBR), using 1 Gy, has demonstrated to be well tolerated in dogs with relapsed lymphoma,

but with no significant clinical response.<sup>405</sup> Higher doses of TBR (11-14,7 Gy),<sup>406,406</sup> after induction chemotherapy and autologous bone marrow transplant, did not reduce the recurrence of lymphoma. Furthermore, higher doses of TBR increased acute and delayed toxicities. Encouraging results were obtained with half body radiation and a variant of CHOP chemotherapy, with obtained median remission duration of 1126 days and median survival time of 1131 days, associated with haematological and GI toxicity. The true statistic power of this study is questionable due to the low number of dogs enrolled (n=13), and mixture of stages and immunophenotypes.<sup>407</sup> Total Skin Electron Therapy (TSET) with 6 Gy is described in a dog with Mycosis fungoid resistant to chemotherapy achieving a long complete remission (19 months), suggesting an eventual role of TSET in the palliative treatment of cutaneous lymphoma in dogs.<sup>408</sup>

The use of autologous Peripheral Blood Hematopoietic Cell Transplantation (PBHCT) in dogs has been described in the treatment either for T-cell and B-cell lymphoma after myeloablative therapeutic. The medians Disease Free Interval (DFI) for the dogs that did not relapse before PBHCT and were in CR at the time of PBHCT were 184 days for T-cell and 271 days for B-cell. The overall survival post-PBHCT was 240 vs. 463 days, for T and B-cell, respectively.<sup>409,410</sup>

### *1.6.5 Immunotherapy*

Exploiting the ability of the immune system to eradicate cancer has been a long-held goal of oncologists. Nevertheless, it constitutes a big challenge for several reasons. Systemic and intra-tumoral cytokine deregulation is associated with the production of strong immunosuppressive cytokines, such as interleukin 10, interleukin 6 and Transforming Growth Factor  $\beta$  (TGF- $\beta$ ). Also, IL-10 and TGF- $\beta$  can inhibit the function of Cytotoxic T Lymphocytes (CTLs), generating T regulatory cells, compromising immune response and creating tumour resistance to apoptosis.<sup>411,412</sup>

An effective anti-tumour immune response is mounted after the processing and presentation of Tumour-Associated Antigens (TAAs) by Antigen Presenting Cells (APC's) to T-cells followed by T-cell activation and proliferation. The primary obstacle opposing this physiologic response is immune tolerance,<sup>413</sup> for that contributes the immune-editing of cancer, where selected tumour cells, that comprise the lymphoma, may have very few TAAs recognizable to T-cells<sup>414</sup> and/or the immunosuppressant effect of the tumour microenvironment, creating a highly defensive barrier that limits the efficiency of an immune response. Successful immunotherapies are the ones who can avoid or decrease immune tolerance to re-establish physiologic host anti-tumour immunity.<sup>411</sup>

It is now more than 20 years, that the discovery and addition of a human/murine, chimeric anti-CD20 Monoclonal Antibody (MAb) to CHOP chemotherapy protocol, in human patients with various CD20-expressing lymphoid malignancies became a historic landmark in the treatment of indolent and aggressive forms of human B-cell non-hodgkin lymphoma.<sup>10</sup>

This immunochemotherapy treatment has become widely used and designated by R (Rituximab®)-CHOP therapy. Meanwhile, several monoclonal antibodies anti-CD20 are available nowadays, with proven efficacy and well-defined safety profiles. The addition of these antibodies to existing chemotherapy protocols, such CHOP changed the treatment landscape for patients, not only prolonging response rates, progression free-survivals and extending overall survival in several human B cell malignancies, namely FL, DLBCL, CLL, and MCL, with acceptable toxicity.<sup>10,415</sup>

In the veterinary field, In 2014, a canine-specific anti-CD20 antibody (Blontress®) received full licensure in the United States of America, but unpublished studies failed to demonstrate clinical efficacy and specificity of the antibody, leading to its withdrawal from the market.<sup>416</sup> A pre-therapeutic approach using immunotherapy with a chimeric human/murine monoclonal antibody against CD20 was tested in dogs, but no reactivity was noticed, discouraging further attempts to use it.<sup>417</sup> Further studies have been conducted to develop and evaluate an anti-canine CD20 antibody, but to date, it is not available to be used in clinical settings.<sup>418,419</sup>

For T-cell lymphoma, also in 2014, a caninized MAb (Tactress®) was developed to treat canine T-cell lymphoma. The target of this MAb, CD52, was evaluated in conjunction with cytotoxic chemotherapy (CHOP-based protocol and Lomustine) for treatment of canine T-cell lymphoma, but does not seem to add significant clinical response, probably because its not specific for the CD52 target as expected.<sup>420</sup> The current status of this antibody development is not known.<sup>416</sup> Further antibodies and antibody conjugated molecules such as MAb B5 (specific for canine leucocyte antigen DR (DLA-DR))<sup>421</sup> are under clinical development.

Peruzzi and colleagues (2009), in a non-randomized, non-controlled study, evaluated the therapeutic use of a vaccine against canine telomerase reverse transcriptase (TERT) in combination with a multidrug chemotherapy (COP) in canine B-cell lymphoma. On treated dogs, vaccine promoted 93% of specific immune response, resulting in a significant benefit in prolonging Median Survival Time (MST) in the vaccinated group compared with the non-vaccinated, 76,1 vs. 29,3 weeks, respectively<sup>384</sup>; however the low number of dogs treated and the small dimension of an historical control group, can compromise the evaluation of the true clinical benefit of it's use. More recently, the use of canine TERT immunization by electroporation in dogs affected by DLBCL, confirmed the specific antibody response, its safety and improved overall survival, when added to a CHOP protocol.<sup>383</sup>

Heat-shock proteins are chaperon proteins produced under stress conditions and their synthesis is high in cancer cells, promoting a stimulus for NK-cells targeting cell death.<sup>422</sup> Its use on a randomized, double-blinded placebo-controlled trial, evaluated the role of active immunization of dogs with DLBCL treated with a CHOP variant vs. CHOP variant plus autologous Heat Shock Protein Peptide Chaperone (HSPPC)-based vaccine.<sup>382</sup> Vaccinated dogs had longer median first and second Progression-Free Intervals (PFI) (304 vs. 41 days and 127 vs. 32 days, respectively). The extension of this therapy to a larger number of dogs with B cell lymphoma subtypes showed very promising results, namely confirming in vaccinated animals increased survival rates, compared with only CHOP based chemotherapy, for diffuse large B-cell lymphoma and nodular marginal cell lymphoma. For follicular lymphoma, lack of immunotherapy administration was the only variable significantly associated with increased risk of tumour-related death.<sup>423</sup> This immunotherapeutic treatment registered under the name APAVAC® is widely commercially available.

Chimeric Antigen Receptor (CAR) T-cells, are allogeneic or autologous isolated T-cells that are genetically modified to express a synthetic structure, combining an extracellular binding domain, with activating signalling domains from the T-cell-receptor complex. They preclude a designated personalized form of immunotherapy and have emerged in recent years as a promising treatment for patients with advanced B-cell cancers, although still with largely associated side effects, specially primarily cytokine release syndrome and neurologic toxicity.<sup>424</sup> The mechanism of action of CAR-cells is mediated by cytotoxic cell death after recognition of specific cell-surface antigens through the extracellular domain.<sup>425</sup> The specificity and cytotoxic effect of dog's CAR T-cells have been evaluated on a model of cDLBCL. Five dogs with DLBCL that were treated with CAR T-cells were able to kill canine CD20<sup>+</sup> lymphoid cells. Circulating CAR T-cells were detectable post-infusion, although, induction of dog anti-mouse antibodies promoted CAR T-cell loss culminating in antigen escape and development of CD20-disease.<sup>426</sup> CAR T-cell addition to CHOP based chemotherapy demonstrated improve outcome, compared to historical controls<sup>427</sup> in cL. However, despite important progresses, CAR T-cell therapy is still in its early days and has significant challenges, such as the complexity, toxicity and costs associated with this technology.<sup>428,424</sup>

Despite interesting results with the developed immunotherapies to treat cL, veterinary medicine is far beyond from human medicine, mainly due to the fact that immune cells and their functionality in dogs are less well described, principally due a lack of canine-specific reagents to detect surface markers and specific cytokines to be used in isolation and expansion of their immune cells.<sup>429</sup>

The use of non-specific cytokines such as type I interferon with anti-proliferative and immunomodulatory capacities have also been evaluated in the treatment of cL, but there is a lack of solid field studies with adequate number of patients to prove its efficacy. With poor evidence and high cost, its use in veterinary patients has been very limited.<sup>430</sup>

#### *1.6.6 Small molecules*

The study and acquired knowledge in malignant cells signalling pathways and intracellular mechanisms revealed numerous potential molecular targets, such as Tyrosine Kinases (TKs). The clinical use of Small Molecules Inhibitors (SMI), which critically block signalling pathways, promote apoptotic mechanisms or modulate the tumour microenvironment, may result in significant clinical benefit for patients. Canine lymphomas have shown to have deregulation of TKs, collaborating in multiple signal transduction pathways that regulate cell survival, activation, proliferation and differentiation.<sup>431</sup> In cL, several studies evaluated the overexpression or deregulation of TKs and the use of small molecules inhibitors as therapeutic approach, but to date, despite the presence of CD117 (stem cell receptor or proto-oncogene c-KIT)<sup>431</sup> and Vascular Endothelial Growth factor (VEGF)<sup>432</sup> expression in high-grade T-cell lymphomas, there is no strong evidence to substantiate the specific use of SMI's in the clinical setting.<sup>320,433-438</sup>

A brief description of documented studies evolving SMI's tested for use in cL is summarized in the following paragraphs.

Exportins are a class of cell transport proteins responsible for nuclear export of proteins, namely tumour suppressor and growth regulatory proteins.<sup>439</sup> Verdinexor® (KPT-335) is an orally available slowly reversible inhibitor of Exportin-1, also known as XPO1, the main mediator of nuclear export, responsible for the binding and nuclear export of a large variety of important tumour suppressor genes.<sup>416,440</sup> A phase II open-label field study of the safety and anti-tumour activity of Verdinexor® in client owned dogs with B and T-cell lymphomas reported an ORR of 37% (71% in dogs with T-cell lymphoma), with mean remission duration of 18 days. Adverse effects were mainly gastrointestinal, namely low grade anorexia, weight loss, weakness and hepatopathy.<sup>440</sup>

Phosphatidylinositol 3-kinase (PI3K) is an important intracellular kinase signalling hub and there's evidence of PI3K pathway deregulation in canine lymphoma.<sup>441</sup> A non-randomized, open label evaluation of an oral PI3Kd inhibitor was used in a phase I/II study in dogs with B and T-cell lymphoma. At the optimal biologic dose, the ORR was 77% (3% CR) with an overall median time to progression of 25,5 days. Activity was observed in both B-cell and T-cell lymphomas, associated with gastrointestinal and hepatic toxicities.<sup>442</sup>

Rabacfosadine (Tanovea®) is a pro-drug of the guanine nucleotide analog 9-(2-phosphonylmethoxyethyl) guanine (PMEG). This molecule after undergo metabolism is a potent inhibitor of the major DNA polymerases restraining DNA synthesis, resulting in S phase arrest and induction of apoptosis in lymphoid tumour cells.<sup>443</sup> When used, improved clinical benefit has been seen in B-cell malignancies. Described clinical response rates of 50% to 85% are dependent of tumour immunophenotype.<sup>395</sup> When used in alternating combination with doxorubicin, ORR was 84% (68% CR), and the overall median PFI was 194 days, results very similar to those reported for conventional CHOP-based chemotherapy.<sup>379</sup> In a prospective trial for canine cutaneous T-cell lymphoma, overall response rate was 45% and biologic response rate of 64%. The median PFS of all dogs was 37,5 days.<sup>444</sup> Gastrointestinal and hematologic adverse effects were mild but a cumulative dermatopathy can occur and, rarely, idiosyncratic, delayed pulmonary fibrosis can also be observed.<sup>416</sup> In the setting of relapsed or refractory lymphoma Rabacfosadine potentiated with asparaginase promoted 41% or CR and a median PFS of 144 days with no increased toxicity regarding comparable studies.<sup>445</sup>

The tyrosine kinase receptor c-kit, also referred to as stem cell factor receptor, proto-oncogene KIT or CD117, is a trans-membrane glycoprotein that shows structural similarities with the Platelet-Derived Growth Factor (PDGFR)<sup>446</sup> Both c-Kit and PDGFR have been demonstrated to be strongly expressed in canine high grade T-cell lymphomas,<sup>320,447,448</sup> epitheliotropic lymphoma<sup>449</sup> and intestinal T-cell lymphoma.<sup>450</sup> Masitinib is a potent inhibitor of KIT receptor and platelet-derived growth factor receptor (PDGFR).<sup>451</sup> The clinical use of Masitinib (Masivet®) in cETCL led to an overall response rate of 70% with 20% of complete remission lasting in median for 85 days. Fifty per cent of the dogs went into partial remission for a median of 60,5 days.<sup>452</sup> It also can reverse doxorubicin resistance by inhibition of P-gp of lymphoid cells in vitro.<sup>453</sup> Toceranib phosphate (Palladia®) is a tyrosine kinase inhibitor that targets PDGFR, VEGFR and KIT. When evaluated to use in multidrug resistant relapsed lymphomas, 40% of patients showed partial response with a median PFI of 94 days.<sup>448</sup> Both molecules showed good tolerance and minimal toxicity.

On going research in preclinical models keeps evaluating new molecular targets and their inhibitors, such as Survivin,<sup>438</sup> Pevonedistat<sup>454</sup> an inhibitor of the NF-kB pathway and Panobinostat,<sup>455</sup> with promising results in cell culture and murine models.

### *1.6.7 Specific T cell lymphoma treatment*

With the exception of low-grade/indolent subtypes, dogs with T-cell lymphoma generally have inferior outcomes when compared with dogs with B-cell lymphoma, in treatment

response evaluation to either a single dose of doxorubicin,<sup>456</sup> or to more aggressive multidrug protocols such as CHOP, in the last case, the expected response rate is around 96% , with median overall survival time of 235 days and 323 days.<sup>457</sup> Among dogs with high-grade TCL, only 50 to 67% will have CR when treated with chemotherapy.<sup>161</sup>The lower response of T-cell lymphomas to doxorubicin as single or as part of multiagent protocols can be due to the lower expression of topoisomerase II $\alpha$ , the molecular target of anthracycline, when compared to B-cell lymphoma<sup>321</sup> or to the overexpression of P-gp.<sup>356</sup>

In dogs with T-cell lymphomas the use of “alkylant rich” protocols, either LOPP (Lomustine-Vincristine-Procarbazine and Prednisolone) or VELCAP-TSC (Asparaginase-Vincristine-Cyclophosphamide-Doxorubicin-Actinomycin-D-Procarbazine and Lomustine) (Table 7), gathered an ORRs ranging from 73% to 97% (64%–90% CR), with median PFS of 175 to 431 days and MST of 237 to 507 days, respectively.<sup>377,458</sup> Further studies are necessary to validate the use of these rich alkylant protocols on treatment response and survival advantage over CHOP based protocols.<sup>416</sup>

Table 7: Comparison of clinical response in dogs with T cell lymphoma treated with CHOP vs. alkylant rich protocols.

<b>Protocol</b>	<b>CHOP<sup>457</sup></b>	<b>VELCAP-TSC<sup>458</sup></b>	<b>LOPP<sup>377</sup></b>
Number of dogs	24	70	31
Clinical Response Rate (CRR) (%)	88	72,9	97
1-year survival rate (%)	14	26,8	39
2-year survival rate (%)	5	15,8	25

In the particular case of TZL, the decision to change from a “watchful waiting” to treatment can be reasoned based on obvious signs of progressive disease such as: 1) developing of clinical signs (substage b); 2) rapid progression of lesion’s size (doubling time of <6 months); 3) peripheral blood lymphocyte count >9200/ $\mu$ l; 3) multiple involved sites or lesions compromising functions; 4) development of myelosuppression due to myelophthisis or 5) organ dysfunction due to infiltration.<sup>396</sup>

### *1.6.8 Treatment response evaluation*

Non-Hodgkin Lymphoma in dogs presents a significant response to therapy, with the majority of the animals achieving complete or partial remissions after treatment initiation and consolidation. The presence of disease persistence or relapse often highlights the need to intensify or re-initiate anti-neoplastic therapy, respectively. The actual guidelines to assess

treatment response in dogs affected by lymphoma uses the concept of Overall Response (OR) having into account the concepts of complete and partial remissions, Stable Disease (SD) and Progressive Disease (PD), considering the measurement and behaviour of the so-called target, non-target and new lesions. The peripheral lymphnode measurements with calliper to assess disease burden have proved an excellent methodology. This method has inter and intra-rater reliability being the assessment of remission status or response to therapy independent from variability in different experienced raters' measurements.<sup>459</sup> Also, due to the great prevalence of intermediate to high-grade lymphomas observed in dogs, the clinical re-evaluation is advised to be monthly for 18 months and, after that, every 2 months.<sup>167</sup> The principles and definitions to be used for assessment of disease burden after treatment are stipulated in the Response Evaluation Criteria for Peripheral Nodal Lymphoma in dogs.<sup>167</sup> These guidelines are exclusive for nodal lymphoma excluding all the extra-nodal forms and it will probably be updated in a near future. It is a document of extreme importance contributing to uniform response assessment, with reproducibility, impacting positively the scientific quality of cL research work and clinical trials.<sup>167</sup>

An emerging procedure that has gained importance in the clinical setting is the measure of Minimal Residual Disease (MRD), defined as the residual population of tumour cells that survive during of after treatment.<sup>462</sup> MRD has prognostic impact<sup>463</sup> and can be assessed in several body compartments, such as lymphnode, peripheral blood and bone marrow.<sup>348</sup> Several techniques including Flow cytometry,<sup>464</sup> PARR,<sup>462</sup> quantitative PCR (qPCR) <sup>465</sup> and GeneScan software analysis<sup>466</sup> have been described and it seems that the sensibility of detection improves with the combination of more than one technique.<sup>348</sup> The possibility of being able to monitor MRD in cL would impact actual treatment paradigms. The stratification of patients by molecular disease burden, would lead to treatment intensification in at-risk patients or prompt treatment in molecular disease relapse.<sup>461</sup>

The use of more advanced techniques, out of clinical trials, to evaluate response assessment is also recommended, but in veterinary medicine the access to techniques such as genetic profiling, flow cytometry or advanced imaging (PET/CT) are not widely available or completely standardized in veterinary practice, either due to a lack of human/physical/technological availability or, when available, to the cost can be outside the financial capabilities of most owners.

## 1.7. Prognosis

As previously stated, due to its high incidence and importance as comparative model for hNHL it is one of the most investigated tumours in domestic animals. The permanent research over the last three decades, has added, removed and changed the list of factors (Table 8) affecting prognosis due to several aspects, namely the number and clinical heterogeneity of enrolled dogs and the diversity and nature of the specific end points examined as it influences the results of such outcome analyses.<sup>16,467</sup>

There are factors that became constant and validated all throughout the time: T-cell lymphomas, apart TZL, WHO clinical and WHO substage b disease always respond worse to therapy, with shorter survival times.<sup>396</sup> The WHO clinical stage has modest prognostic information, although it is known that dogs with stage I/II of the disease have better outcomes than later stages (III- V), or substage b, are consolidated predictors of poorer outcomes.<sup>468,469</sup> However, for cL, bone marrow infiltration assessment is not recommended under staging guidelines<sup>167,351</sup> and the actual absence of defined criteria to determine clinical substage<sup>470</sup> can promote doubt in how stage V and substage b designations are currently made.

In the early 2000 the implementation of the WHO classification scheme consolidated the prognostic significance of lymphoma subtypes, namely the increased survival times of indolent forms, such as TZL, MZL versus aggressive forms (DLBCL, PT-NOS, etc.). The prognostic parameters (Table 8), in general, can be categorised as following: 1) factors associated with the patient, 2) factors associated with the host reaction to the tumour and 3) factors related to the tumour. A fourth category may be possibly emerging: geographic localization, representing differences in availability of advanced medical care, rate of early detection and diagnosis, rate of commonly used screening tests, socio- economic factors and differences in tumour subtype distributions among other causes.<sup>471</sup>

In the specific case of nodal high grade T-cell lymphoma, immunotypes are very important to predict prognosis, with tumours expressing CD4<sup>+</sup>/CD8<sup>-</sup>/MHCII<sup>+</sup>, CD4<sup>-</sup>/CD8<sup>+</sup>/MHCII<sup>+</sup> and CD4<sup>-</sup>/CD8<sup>+</sup>/MHCII<sup>-</sup> associated with longer survival times than the immunotype CD4<sup>+</sup>/CD8<sup>-</sup>/MHCII<sup>-</sup>. Immunotype characterized by CD4<sup>+</sup>/CD8<sup>-</sup>/MHCII<sup>+</sup>, CD4<sup>-</sup>/CD8<sup>+</sup>/MHCII<sup>-</sup>, and CD4<sup>-</sup>/CD8<sup>-</sup>/MHCII<sup>+</sup> have associated longer progression free intervals. Based on flow cytometry, both BCL and TCL with MHCII high expression had better prognosis than those with MHC II low expression.<sup>183</sup> It has also been reported that CD4<sup>+</sup> TCL has a less favourable outcome than CD4<sup>-</sup> TCL and that patients with CD4<sup>+</sup>/ CD5<sup>-</sup> TCL had slightly longer survival than those with CD4<sup>+</sup>/CD5<sup>+</sup> lymphoma.<sup>108</sup>

Non-neoplastic lymphnode lymphocyte subpopulations can determine response to treatment and outcome, with patients with higher percentages of CD5<sup>+</sup> and CD8<sup>+</sup> small cells, CD5<sup>+</sup>/large CD21<sup>+</sup> cell ratio or decreased CD4<sup>+</sup>/CD8<sup>+</sup> ratio having inferior disease progression times.<sup>472</sup>

Cancer microenvironment plays a pivotal role in tumour survival and progression. Its cellular components are highly heterogeneous and include almost all immune cell types, including T cells (CD8<sup>+</sup>, CD4<sup>+</sup> and regulatory), macrophages, Dendritic Cells (DCs), Natural Killer (NK) cells, B-cells, mast cells among others, with the relative percentages and phenotypes varying considerably between tumour types and patients within the same tumour type. This complexity provides a “fingerprint” for the tumour microenvironment and can be highly correlated with clinical outcome.<sup>473</sup> Regulatory T-cells, identified by its expression of the Forkhead Box Transcription Factor 3 (FOXP3<sup>+</sup>) in addition to the classical markers CD4<sup>+</sup> and CD25<sup>high</sup>, play an important immunosuppressive effect in anti-tumour response.<sup>473,467</sup> In general, in B-cell tumours the levels on non neoplastic FOXP3<sup>+</sup> cells, when compared to healthy dogs, can be increased<sup>474</sup> in lymph nodes of dogs with DLBCL. However, FOXP3 when highly expressed they have a significant negative impact on PFS and OS in canine T-small-cell intestinal and B-cell lymphoma<sup>475,476</sup>, and are inversely correlated with the level of cytotoxic T-cells, decreasing during chemotherapy.<sup>474</sup>

**Table 8:** Described prognostic factors for lymphoma in the dog.

Parameter	Comments	Level of association		References
		Strong	Modest	
<b>Patient related</b>				
Sex	Less favourable for females and neutered females		☐	477,163
Age	Older		☐	478
Weight	Smaller dogs at diagnosis Favourable		☐	12,479,480,467
Pre-treatment with steroids	Unfavourable	☐		480,481
<b>Factors related to the tumour</b>				
WHO stage	Stage V negative Stages I-II favourable		☐	346,479,482,234
Immunophenotype	B cell favourable Exception T2L favourable	☐		162,483,484,468,158,480,482,478,183
Anatomic location	Hepatosplenic, Intestinal (high grade T) cutaneous and leukemic phase unfavourable Cranial mediastinal lymphadenopathy	☐		195,196, 175,176,185,234,241
Histotypes	High to intermediate grade unfavourable Low grade and indolent favourable	☐		17,484,269,130,18,233
Proliferation markers	Contradictory results		☐	485-488
Gene Expression Profiles	Some signatures more favourable	☐		85,489,86,490,491,325
Minimal residual disease	Relative importance since there aren't, yet, curative approaches		☐	463,492-494,348
RNA disruption	RDI>0,6 favourable		☐	495
FOXP3+	Unfavourable	☐		467,476
CD25 expression (DLBCL)	Unfavourable		☐	496
<b>Factors associated with the reaction from the host to the tumour</b>				
Sub-stage	Sub-stage b not favourable	☐		12,468,158,346,497
Anaemia	Unfavourable	☐		253,347,253,347,28,302,497
Monocytopenia	Unfavourable		☐	28
Neutrophilia	Unfavourable	☐		347,227
NLR	Longer PFS <9,44 Longer OS < 11,44		☐	302,497
LMR	Longer PFS>1,43 Longer OS>1,43		☐	302,498,28
PNR	Early progression>0.032		☐	497
Thrombocytopenia	Unfavourable		☐	227
Albumin	Unfavourable if low		☐	499,196
Chemotherapy induced neutropenia	VCOG Grade III or IV favourable		☐	500,501

## **2. The pertinence of the investigation.**

### **2.1 Prognostic factors for canine diffuse-large B cell lymphoma**

In veterinary oncology, the clinical studies on specific entities of cNHL as defined by the WHO classification scheme are very few and recent, dating from the last decade.<sup>15</sup>

Most of the prognostic factors described for canine lymphoma, were determined by studies including mixed lymphoma subtypes, tumour stages, diagnostic and treatment variations, limiting the value of the studies and the significance of the results obtained.<sup>16</sup>

Diffuse-large B-cell lymphoma in the dog has been receiving important distinction in the last decades as the comparative model for the human counterpart.<sup>502</sup> This fact, lead to the development of intense research on cDLBCL and on its comparative aspects, namely at molecular, genetic, immunologic and histologic level. The clinical comparative aspects are less investigated namely, response to treatment and prognostic.<sup>86,325,503</sup> In consequence, more clinical studies on DLBCL are needed.

Animal owners support the costs of the veterinary treatments, since pet health insurance in Portugal is a resource, despite its existence, scarcely used. Cancer diagnosis and treatment is costly and can strongly condition owner decision to treat. Prognosis and response to treatment are then fundamental for owner's decision to treat.

The availability of cost efficient, fast and reliable prognostic markers is of extreme importance, since it can impact veterinary-client communication, client understanding of potential disease outcomes and, consequently, their decision making whether to treat their pet with the associated financial investment and ethical decision making required.<sup>504</sup>

### **2.2 Why peripheral blood cell ratios?**

In human oncology, peripheral blood cell counts and their derived ratios, specially Neutrophil-to-Lymphocyte and Lymphocyte-to-Monocyte ratios have shown to be strong predictors of prognosis in several malignancies, namely in diffuse large B cell lymphoma.<sup>26</sup>

Patient outcome and prognosis can vary with geographic localization, demographics, socio-economic factors and differences in tumour subtype distributions among other causes.<sup>471</sup>

A small number of studies have evaluated peripheral blood cell counts and derived ratios in canine lymphoma, but there is a lack of uniformity in patient enrolment, staging procedures or treatments offered.<sup>302,227, 498, 505</sup>

In Portugal, there are a very small number of studies on canine lymphoma, but none assessing clinical aspects namely, response to treatment, clinical outcome and/or prognostic.<sup>54,66,400,412,455</sup>

## **2.3 Rationale to evaluate vector-borne diseases in dogs with lymphoma**

There are a few case reports of the presence of canine vector-borne agents in dogs<sup>35-37,135,136</sup> and humans,<sup>39,506</sup> diagnosed with lymphoma.

Lisbon is an endemic area for vector-borne diseases (VBD).<sup>507,508</sup> Geography has a relevant importance in the existence and pattern of vector-borne diseases, contributing to concomitant morbidities, affecting significantly diagnostic and/or laboratory results, treatment outcome and associated prognostic in patients with cancer.<sup>471</sup>

Vector-borne diseases in dogs are associated with several clinicopathological abnormalities. Infection with organisms from the genus *Leishmania*, *Ehrlichia* and *Anaplasma* in dogs can cause anaemia, thrombocytopenia and leukogram alterations, namely leukopenia.<sup>509,510</sup> Anaemia, monocytosis, neutrophilia and thrombocytopenia are also hematologic features of dogs infected with the genus *Bartonella*.<sup>510</sup>

The pertinence on the cancer promoting potential of *Leishmania infantum* has been also pertinently discussed.<sup>511</sup>

This preliminary and complementary investigation enabled us to have an insight of how common are CVBD in dogs with lymphoma in the Lisbon Metropolitan Area and how these infections could, eventually, impact their haematological values, lymphoma subtype and possible role in lymphomagenesis.


### 3. Objectives

This research project by using an uniform and state-of-the-art diagnosis, staging, standard of care homogeneous therapeutic approach and long clinical follow up of naïve cases of canine non-Hodgkin lymphoma, aimed:

- To determine the frequency of specific canine vector-borne agents (*Leishmania infantum*, *Ehrlichia canis*, *Anaplasma phagocytophilum* and *Bartonella henselae*) infection in dogs suffering from lymphoma, in the Lisbon Metropolitan Area and evaluate the eventual association with lymphomagenesis, alterations in peripheral blood cells counts and lymphoma subtypes,
- To evaluate the prognostic impact at diagnosis of sex, neuter status, clinical stage, haemoglobin concentration and specific peripheral blood cells counts (neutrophils, platelets and red blood cells) and derived ratios (NLR, LMR, PNL, PLR) in dogs with stage II/IV DLBCL treated with a CHOP19 protocol.

## **5. Canine lymphoma and vector-borne diseases: Molecular and serological evaluation of a possible complicity**

# Canine lymphoma and vector-borne diseases: Molecular and serological evaluation of a possible complicity

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## Abstract

Lymphoma is the most common haematological malignancy in dogs and its aetiology is largely unknown. The presence of canine vector-borne agents (CVBD) in lymphoma tissues has been described and its causative effects questioned. We intended to evaluate the presence and extent of *Leishmania infantum*, *Ehrlichia canis*, *Anaplasma phagocytophilum* and *Bartonella henselae* infection in dogs with lymphoma. Sixty-one dogs, living in the Lisbon metropolitan area, with a diagnosis of lymphoma were enrolled. Immunofluorescence assays were used to detect serum IgG's. The presence of DNA from CVBD agents in tumour tissue was assessed by PCR. All dogs tested negative for *B. henselae*, *A. phagocytophilum* and *E. canis* by both serology and PCR. Regarding *L. infantum*, 8.2% (n = 5) of the dogs had a positive serologic result. *L. infantum* DNA was detected in two samples of diffuse large B-cell lymphoma (DLBCL). These results show an increased, but not significant, seropositivity (8.2% vs 7.9%) and molecular detection (3.3% vs 1.2%) for *L. infantum* in dogs with lymphoma, when compared to the reported canine population in the same geographical area. We could not identify an association between lymphoma and *E. canis*, *A. phagocytophilum*, *B. henselae* or *Leishmania infantum* infection in the studied population. Nevertheless, further studies, following dogs through their CVBD disease evolution, are worthwhile and may help clarify a possible role of CVBD agents in lymphomagenesis.

## KEYWORDS

diffuse large B-cell, dog, infectious diseases, Leishmania, lymphoma, vector-borne

## 1 | INTRODUCTION

Lymphoma is one of the most common canine cancers, estimated to account for up to 83% of all canine haematological malignancies.<sup>1</sup> Although its aetiology is largely unknown and believed to be multifactorial, several reports have associated canine lymphoma (cL) development with environmental<sup>2,3</sup> and genetic<sup>4,5</sup> causes and autoimmune,<sup>6,7</sup> hemoparasitic<sup>8-11</sup> and viral<sup>12</sup> diseases.

The exact mechanism by which chronic inflammatory processes induce lymphomagenesis is still unclear, but persistent

immunosuppression,<sup>13</sup> epigenetic events<sup>14</sup> and relevant association with immunosenescence<sup>15</sup> have all been associated with lymphoma development.

Vector-borne (VB) diseases are a One Health problem, and pets are both important reservoirs and natural models for human-related infections.<sup>16</sup> VB agents promoting chronic inflammation can change host epigenetic pathways, induce gene and /or protein expression abnormalities and promote immunosuppression.<sup>17</sup>

Infectious agents can also induce chronic B-cell stimulation, suppress T-cell activity and predispose an infected animal to lymphoma

or alter the pathogenesis of lymphoma in a host.<sup>18</sup> In humans, an association between *Leishmania* spp.<sup>19</sup> and *Borrelia burgdorferi*<sup>20-22</sup> infection and cutaneous lymphoma has been hypothesized. Reports of the concomitant presence of *Leishmania infantum* and lymphoma in dogs<sup>10</sup> and humans<sup>23</sup> do exist, as well as the acknowledged *Leishmania*'s oncogenic potential, either by depletion of Th1 response or by promoting Th2 cytokine environment response, leading to the proliferation of tumour cells or vice versa<sup>23</sup> (Figure 1). Also, the presence of a lymphoma, and the associated treatment with chemotherapy can lead to immunosuppression, which will contribute to Leishmaniasis progression, causing active disease with associated comorbidity. In the case of *Bartonella* spp., acute or chronic manifestations of infection can occur, sometimes resembling neoplastic granulomatous lymphadenopathy<sup>24</sup> and its presence is described in neoplastic tissues from dogs with lymphoma<sup>11</sup> and hemangiosarcoma.<sup>25</sup>

Ticks, mosquitoes, sand flies and fleas are major vectors<sup>26</sup> and, in some cases, several infectious agents can coexist on the same vector, as already described for the concomitant presence of Anaplasmataceae, *Borrelia* spp., *Bartonella* spp. and *Rickettsia* spp. in *Ixodidae* ticks<sup>27</sup>.

In Portugal, as in many Mediterranean basin countries, vector-borne agents commonly infect pet dogs.<sup>28</sup> In the last Portuguese national serological survey on canine vector-borne diseases (CVBD),<sup>29</sup> using a case-control study, it was concluded that dogs are at risk of developing an infection, among other agents, with *Ehrlichia canis*,

*Anaplasma* spp. and *Leishmania infantum*. The evaluation of exposure to these pathogens in healthy vs symptomatic/suspected dogs was detected in 4.1% vs 16.4%, 4.5% vs 9.2% and 4.3% vs 25.2%, respectively. The same study also shows that in Lisbon metropolitan area seropositivity is even higher for *Ehrlichia canis* (6.3% vs 19.4%), *Anaplasma phagocytophilum* (6.3% vs 11.5%) and *Leishmania infantum* (7.9% vs 30.2%) in healthy vs symptomatic dogs, respectively.<sup>29</sup>

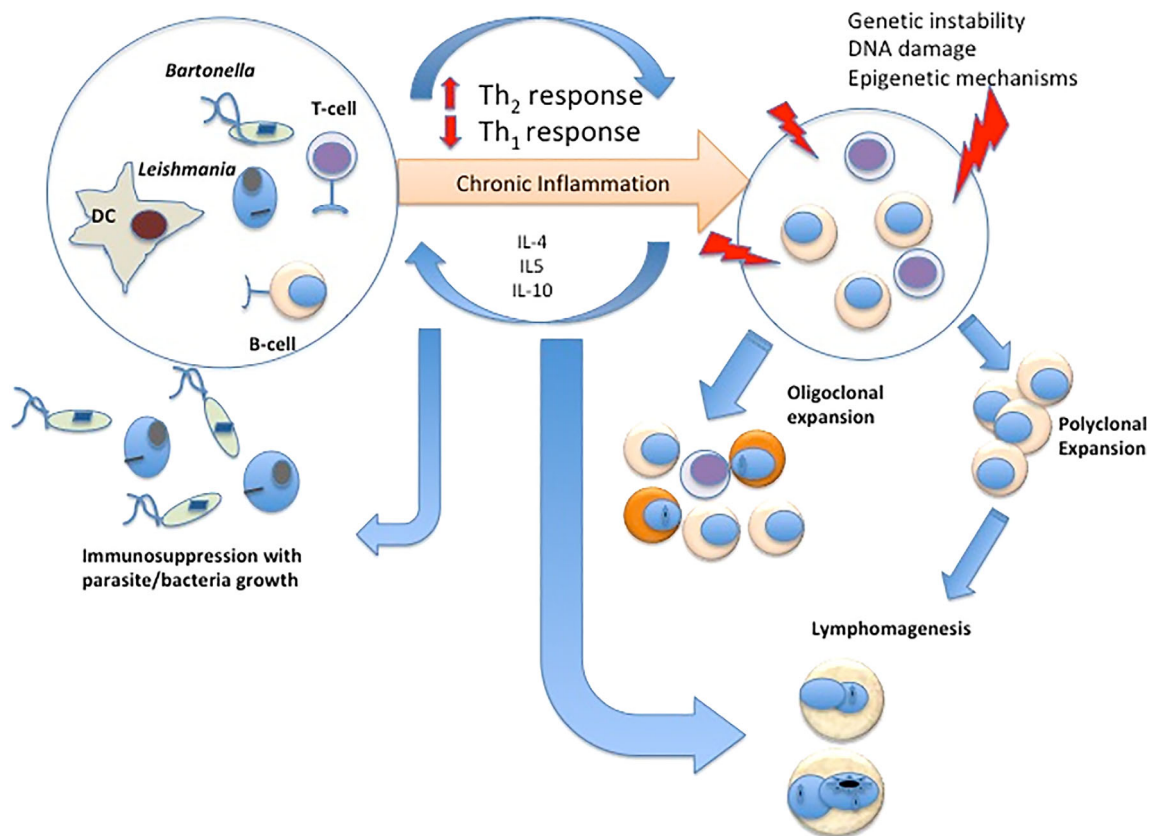
## 2 | OBJECTIVES

Based on what was described, and the potential implication of canine infections caused by vector-borne pathogens and cancer development, our study aimed to evaluate if there was any association between dogs suffering from multicentric lymphoma and infection by *Leishmania infantum*, *Ehrlichia canis*, *Anaplasma phagocytophilum* and *Bartonella henselae*.

## 3 | MATERIAL AND METHODS

### 3.1 | Patients and samples

The present study included 61 treatment naïve dogs with a histological diagnosis of lymphoma, which were presented for treatment at a



**FIGURE 1** Diagram of possible underlying mechanisms that can contribute to infection-related lymphomagenesis<sup>13-15,17</sup> [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

referral hospital (omitted for blind review) between 2010 and 2014. All the animals were originally from the Lisbon Metropolitan area. All dogs were subjected to a full clinical examination, epidemiological data collection (age, breed, gender, reproductive status) and lymphoma-staging procedures, including total blood count with blood smear, biochemistry panel and abdominal ultrasound. An incisional biopsy from one affected lymph node (popliteal) was obtained for diagnostic purposes. The biopsies were submitted to an external commercial laboratory for routine histopathology and immunohistochemistry, and tumours were classified according to the WHO classification scheme for canine lymphoma.<sup>30</sup> For molecular diagnosis, a biopsy specimen was conserved in RNAlater solution (Sigma-Aldrich) and frozen at  $-80^{\circ}\text{C}$  until further processing.

For CVBD serology testing, serum samples were obtained from blood collected for staging purposes, before any treatment. All samples were frozen at  $-80^{\circ}\text{C}$  before processing.

Two historical populations of dogs, published on national surveys for canine VB diseases in Portugal were used as control groups for serologic<sup>29</sup> and molecular<sup>31</sup> detection of CVBD.

### 3.2 | Serological analysis

Immunofluorescence assays (IFA) were performed to test serum samples for the presence of antibodies against *L. infantum* (*Leishmania infantum* IFA canine IgG kit, Fuller), *E. canis* (*Ehrlichia canis* IgG IFA kit, Fuller), *B. henselae* (*Bartonella henselae* IgG IFA kit, Fuller) and *A. phagocytophilum* (in house assay, Portuguese National Institute of Health Doutor Ricardo Jorge-CEVDI/INSA). Fuller commercial kit assays were performed according to manufacturer's instructions. *A. phagocytophilum* assay was performed according to CEVDI/INSA procedure using *A. phagocytophilum* Webster strain as a source of antigen as previously described by Santos et al., (2009).<sup>32</sup>

Serology tests were considered positive for *B. henselae*, *A. phagocytophilum* and *E. canis*, with titers of IgG  $\geq 128$  and IgG  $\geq 80$  for *L. infantum*.

### 3.3 | DNA extraction

Lymph node fragments obtained from biopsies were defrosted at room temperature, and DNA was extracted from 20 to 25 mg pieces using the commercial DNeasy Blood and tissue kit (Qiagen), according to manufacturer's instructions. DNA was eluted in 100  $\mu\text{L}$  of sterile deionized water and stored at  $-20^{\circ}\text{C}$  before PCR.

### 3.4 | Polymerase chain reaction

All DNA samples were first amplified for a canine  $\beta$ -actin gene fragment, with the primers described by Maia et al (2010),<sup>33</sup> to assess successful DNA extraction and exclude the possibility of PCR inhibitors.

A conventional PCR was performed to amplify a 447 bp fragment of *Leishmania donovani* s.l. minicircle kinetoplast DNA with primers MC1 and MC2, as described by Cortes et al (2004).<sup>34</sup>

For *Anaplasma* spp. and *Ehrlichia* spp. detection, a conventional PCR was prepared to amplify a 16S rRNA gene fragment with primers GEPs and GEP, as previously described by Maggi et al (2006).<sup>35</sup>

*Bartonella* genus screening was made by conventional PCR with primers 325s and 1100as targeting a 16S-23S ITS region fragment, as described by Maggi et al (2006).<sup>35</sup>

## 3.5 | Statistical analysis

Data was entered into Microsoft Excel and statistical analysis was performed using SPSS Statistics 26 software. Chi-squared and Fisher's exact tests were used to assess differences between studied groups. Results were considered statistically significant when  $P < .05$ .

## 4 | RESULTS

Sixty-one dogs with lymphoma were included in the present study. Dogs' characteristics are described in Table 1.

The dogs' median age was 10.4 ( $\pm 0.41$ ) years, with 41% (25/61) of the cases diagnosed in animals with more than 10 years of age, and 57.4% (35/61) in dogs between 5 and 10 years. Intact males and neutered females together constituted 75.4% of all the present cases, with 42.6% (26/61) and 32.8% (20/61), respectively.

Crossbreed dogs were the most common represented breed, contributing almost 24.6% (15/61) of the studied population. The second most affected breed was Rottweiler representing 13.1% (8/61), followed by Boxer 8.2% (5/61), Golden Retrievers and Cocker Spaniels with 6.6% each (4/81) and Labrador Retrievers and Doberman with 4.9% each (3/61).

Lymphomas were classified according to WHO criteria<sup>30</sup> (Table 1). High-grade lymphomas accounted for 80.4%, consisting of diffuse large B-Cell lymphoma (DLBCL) with 60.7% (37/61), peripheral T cell-not-otherwise-specified (PT-NOS) with 16.4% (10/61) and Burkitt Lymphoma (BL) with 3.3% (2/61). In the category of low-grade lymphomas (19.6%), follicular lymphomas (FL) were the more prevalent accounting for 9.8% (6/61) of the cases. B cell neoplasms accounted for 83.6% (51/61) of all lymphomas and T-cell for 16.4% (10/61).

Regarding immunofluorescence results, all the 61 dogs were negative for *E. canis*, *A. phagocytophilum* and *B. henselae*, with IgG antibody titers below 128. For *L. infantum*, 8.2% (5/61) of the dogs were positive, with antibody titers  $\geq 80$ , two dogs with a titre of 320 and three of them with titre 2560.

Of the *L. infantum* seropositive dogs, 60% (3/5) were aged between 5 and 10 years; the two remaining dogs were over 10 years. The two dogs with a PCR positive for *L. infantum* were 7 and 14 years old.

Breed	N	%	Age	N	%
Cross	15	24.6	≤5y	4	6.6
Rottweiler	8	13.1	>5- ≤10y	32	52.5
Boxer	5	8.2	>10 y	25	41.0
Golden	4	6.6			
Cocker	4	6.6	Gender	N	%
Labrador	3	4.9	MN	4	6.6
Doberman	3	4.9	FN	20	32.8
Serra da Estrela	3	4.9	M	26	42.6
Pitt bull	2	3.3	F	11	18.0
Sharpei	2	3.3			
Fox Terrier	2	3.3	Lymphoma subtype	N	%
American Staffordshire	2	3.3	High Grade	49	80.4
French Bulldog	1	1.6	DLBCL	37	60.7
Bichon Maltese	1	1.6	PT-NOS	10	16.4
Yorkshire Terrier	1	1.6	Burkitt	2	3.3
German Shepherd	1	1.6	Low grade	12	19.6
Basset Hound	1	1.6	Follicular	6	9.8
Poodle	1	1.6	Mantle cell	1	1.6
Lhasa Apso	1	1.6	Lymphoplasmacytic	2	3.3
West Highland Terrier	1	1.6	Small cell	3	4.9
	61	100.00			

Abbreviations: DLBCL, diffuse large B-cell lymphoma; F, female; FN, female neutered; M, male; MN, male neutered; N, number; PT-NOS, peripheral T-cell lymphoma-not otherwise specified; y, years.

**TABLE 1** Patient's characteristics and lymphoma subtypes

	IFA			PCR		
	POS	NEG	% POS	POS	NEG	% POS
<i>Leishmania infantum</i>	5	56	8.2	2	59	3.3
<i>Ehrlichia canis</i>	0	61	0.0	0	61	0.0
<i>Anaplasma phagocytophilum</i>	0	61	0.0	0	61	0.0
<i>Bartonella henselae</i>	0	61	0.0	0	61	0.0

Abbreviations: IFA, immunofluorescence assay; NEG, negative; PCR, polymerase chain reaction; POS, positive.

**TABLE 2** IFA and PCR results for CVBD agents in dogs with lymphoma

Breed distribution to seropositivity for *L. infantum* included three pure breed (3/5) and two (2/5) crossbreed. On molecular detection, one of the two positive dogs was pure breed (Lhasa Apso) and the other Crossbreed.

When evaluating seropositivity for *L. infantum* and lymphoma subtype, 60% (3/5) of the dogs had DLBCL and the remaining 40% were distributed in the indolent forms: Mantle cell and small cell lymphoma both with 20%, (1/5) each.

PCR amplification of  $\beta$ -actin gene was positive for all samples, excluding the presence of DNA polymerase inhibitors or DNA fragmentation. No amplification was accomplished for *E. canis*, *A. phagocytophilum* and *B. henselae*.

Molecular and serological data are presented in Table 2.

Comparison between our results and those obtained by Maia et al, (2015)<sup>31</sup> and Cardoso et al, (2012)<sup>29</sup> did not reveal significant differences for *L. infantum* PCR ( $P = .166$ ) or serologic positivity ( $P = .201$ ) for *L. infantum* between domestic healthy dogs and domestic dogs with lymphoma.

## 5 | DISCUSSION

This study aimed to identify evidence of infection of four common CVBD agents in dogs suffering from lymphoma and living in the Lisbon area, Portugal. Canine lymphoma tends to develop more frequently in middle-aged to old animals. The estimated incidence rate

increases to 84 in 100 000 dogs at risk in animals after 10 years of age.<sup>36</sup> The fact that we had a higher percentage of animals between 5 and 10 years could be attributed to particular geographic characteristics of the patients than can involve a higher risk in younger ages such as breed, environmental and genetic factors, among other multifactorial factors that can contribute to lymphomagenesis. Our results do not show a particular association with age and CVBD infection. Because sample size and numbers of seropositive and infected dogs are low, no significant conclusions can be obtained regarding the accurate distribution of age and age-associated CVBD. Nevertheless, this increased risk and development of cL with age, probably reflect the suspected multifactorial aetiology of lymphoma and the time needed for all the genetic, environmental and immune causes to work alone or in combination to accomplished lymphomagenesis, namely a potential chronic vector-borne infection.

The breed distribution falls within that expected, as crossbreed dogs are the most common dog breed in Portugal, constituting 58% of all breeds.<sup>37</sup> These results are aligned with what is described for the high-risk pure-breeds.<sup>36</sup> In a previous study, that evaluated canine breed risk for lymphoma in Europe, we demonstrated that dog breeds in Portugal at risk are the same as reported in other countries. However, in Portugal, Rottweiler and Dobermans were overrepresented and had a significant ( $P < .001$ ) odds ratio of 4.59 and 8.84, respectively, for developing lymphoma.<sup>38</sup> Genetic predispositions have been described as promoting susceptibility to *Leishmania* spp. in certain breeds of dogs, such as Boxers, where the TAG-8-141 allele was associated with susceptibility, whereas the TAG-9-145 allele was protective in the homozygous state.<sup>39</sup> Despite being the third most affected breed by lymphoma in our population, none of the Boxer patients had serologic or molecular positive results for *L. infantum*. Other genetic factors can contribute to individual and/or breed susceptibility to cL being the *SLC11A1* gene (formerly *NRAMP1*) the most studied in canine leishmaniasis; there are reports of either protection or susceptibility, depending on the single nucleotide polymorphism presented by the host.<sup>39-41</sup>

In humans, the type of lymphoma that is associated with the causative infectious agent(s) can be very specific (eg, MALT lymphoma due to *Helicobacter pylori* or cutaneous lymphoma due to *Borrelia* spp.).<sup>18,22</sup> Regarding lymphoma histotype in the present study, when compared with the literature, we obtained similar percentages for high-grade and low-grade/indolent lymphomas, 80.4% and 19.6%, respectively. We found higher percentages than reported by other authors for the specific subtypes of canine lymphoma and similar for Mantle Cell lymphoma (1.6%).<sup>30,42,43</sup> The true incidence of canine indolent lymphomas is still unknown because due to the slow growth and absence of clinical signs, it can pass underdiagnosed until transformed into a high-grade disease.<sup>44</sup> Demography is highly variable between geographic regions and this can strongly contribute to differences in the reported lymphoma subtypes, as it happens in humans.<sup>45</sup> Breed distribution can vary strongly between countries or continents, the pure-breed composition and the high number of crossbreed dogs of our population could also have contributed to the differences in our reported subtypes.<sup>46</sup>

The high number of aggressive forms of lymphoma in this study could be because the studied population was presented to a referral oncology service, and this could have led to a pre-selection bias due to referring veterinarians, choosing to treat less aggressive forms or delay referral, allowing the progression of low-grade subtypes to progress to more aggressive forms (eg, follicular forms which can transform and progress to DLBCL).<sup>47</sup>

*L. infantum* DNA was detected in two samples of DLBCL. However, due to the low number of cases of molecular detection and the high prevalence of DLBCL, this can be more casual than a direct association. Nevertheless, a potential association between *L. infantum* infection and DLBCL development cannot be ruled out. Dogs with lymphoma have acknowledged immunologic dysregulation,<sup>48,49</sup> that may potentially predispose to VB infections.

All the dogs with positive results for *Leishmania* spp. infection (serologic and molecular detection) have been already diagnosed with the disease before lymphoma diagnosis. This, theoretically, indicates that the infection could have helped trigger the lymphoma but, as the dogs were not tested further during the course of the disease, the other way around cannot be completely excluded. In the absence of clinical follow up regarding leishmaniasis, the seropositive dogs included in this study may not be actually infected and the PCR positive results may, simply, reflect a latent infection.

The lack of a control group to assess the actual presence of positivity in dogs without lymphoma and the lack of information about clinical presentation and follow up during the disease, are relevant flaws, that make impossible to state definitely any possible relationship (or not) between *Leishmania* spp. and lymphoma or vice versa.

Serology screening by IFA and DNA amplification by PCR resulted negative for *B. henselae*, *A. phagocytophilum* and *E. canis*. It is described that dogs infected with *Bartonella* spp. do not have or have a low level of seroreactivity on IFA.<sup>50,51</sup> The negative results for *B. henselae* molecular detection could be due to low DNA content in the lymph node samples. This fact could have been potentially overcome if a previous culture in specific enrichment culture media had been done, increasing PCR sensitivity.<sup>50</sup> Interesting as well is the fact that a national survey with more than 1000 dogs<sup>31</sup> failed to identify *Bartonella* spp. by molecular detection. Despite the negative results, *Bartonella henselae* is the species that most affects dogs<sup>24</sup> and the negative results reported in Portuguese dogs, needs to be clarified. Seroreactivity and molecular detection of *Bartonella* have been described in Portuguese cats<sup>52</sup> but, to the best of our knowledge, there is no reported data for dogs in Portugal. Maybe in the dog, the primary niche is outside the lymphatic system making it more difficult to detect in lymph node samples. Nevertheless, a previous report in the United States comparing Golden Retrievers with lymphoma, described 18% of positive results by molecular methods,<sup>11</sup> a similar incidence to matched controls, what somehow corroborates the presence of *Bartonella* spp. in the lymphatic system.

In the present study, the negative results obtained by serology and PCR for *B. henselae*, *A. phagocytophilum* and *E. canis*, in lymphoma samples, can be due to effective use of ectoparasiticides, decreasing

or eliminating the risk of infection by the arthropod vectors<sup>29</sup> and in metropolitan areas, dog park's are usually free of grass, reducing the places where vectors can hide or feed. The absence of molecular amplification for *A. phagocytophilum* and *E. canis* in Golden retrievers affected by lymphoma had already been described.<sup>11</sup> It can corroborate the negative presence of this bacteria in dogs with lymphoma and an assumption that there is no cause-effect association in lymphomagenesis and the described association with *Ehrlichia* spp.<sup>53</sup> could have been just a coincidence.

In the Lisbon general dog population, reports of *Leishmania* spp. serology and molecular detection in peripheral blood described positivity rates of 7.9%<sup>29</sup> and 2.3%,<sup>31</sup> respectively. In our study, we had a slightly increased but not significant ( $P = .201$ ) seropositivity compared with the reported values (8.2% vs 7.9%).<sup>29</sup> Regarding molecular detection, for domestic dogs, our results showed a non-significant ( $P = .166$ ) rate increase (3.3% vs 1.2%),<sup>31</sup> probably due to the fact that lymph node, which has been described as the ideal biological sample for *Leishmania* spp. molecular detection, is one of the preferred internal tissues for this parasite invasion and multiplication.<sup>54,55</sup>

The main limitations of our study are the low number of enrolled dogs, which compromises any statistical power, due to the low incidence of the evaluated diseases, and the lack of a control group of dogs with nonlymphoproliferative cancer, reducing the bias associated with the use of historical control populations. Also, several other local CVBD agents, such as *Borrelia* spp. or *Babesia* spp., which may be implied in lymphomagenesis, were not tested.

The lack of the continuous testing and clinical follow up of the positive dogs for the studied CVBD limits the study, as CVBD can possibly affect treatment plan and lymphoma associated outcome,<sup>23,56</sup> so CVBD agents must be tested as a routine pre-treatment evaluation. Another critical factor to have in consideration is the possible confounding results on diagnostic tests for lymphoma, such as peripheral antigen receptor rearrangements clonality assays, since *Leishmania* spp. and *Ehrlichia* spp. infections can cause monoclonal peaks, influencing a putative diagnosis of lymphoma.<sup>57</sup> In our case series, clonality assessment was performed for all samples (results not showed), being all monoclonal, and in accordance with the previous IHC phenotype. No possible monoclonal peak with a polyclonal background due to *L. infantum*<sup>58</sup> was observed.

With the present study we could not identify any association between *E. canis*, *A. phagocytophilum*, *B. henselae* and *Leishmania infantum* and lymphoma. A more robust prospective study, enrolling a larger number of dogs, followed in the course of their *L. infantum* infection is warranted help to clarify the possibility that this parasitic disease could trigger lymphoma, especially DLBCL in dogs.

#### DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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## **6. Peripheral blood cell ratios as prognostic factors in canine diffuse large B-cell lymphoma treated with CHOP protocol**

# Peripheral blood cell ratios as prognostic factors in canine diffuse large B-cell lymphoma treated with CHOP protocol

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## Abstract

Diffuse large B-cell lymphoma (DLBCL) is the most common haematopoietic tumour in dogs and recognized as clinical model for its human counterpart. Recently, neutrophil-to-lymphocyte (NLR) and lymphocyte-to-monocyte (LMR) ratios have been shown to predict time-to-progression (TTP) and lymphoma-specific survival (LSS) in dogs with DLBCL treated with CHOP-based chemotherapy. We retrospectively evaluated in 59 dogs diagnosed with DLBCL the prognostic value of haematological parameters and derived ratios: NLR, LMR, platelet-to-lymphocyte (PLR) and platelet-to-neutrophil (PNR) ratios for TTP, LSS and associated secondary end-points (time-to-progression-rate [TTPR] and lymphoma-specific survival-rate [LSSR]) as rates at 180 and 365 days. PNR is an independent prognostic marker ( $p \leq .001$ ) for TTPR/180 and 365 days, dogs with a PNR above 0.032 were more likely to progress before 180 days (sensitivity 46.5%, specificity 87.5%,  $p = .004$ ). On univariate analysis, NLR showed a prognostic significance for LSSR/180 ( $p = .006$ ) and LSSR/365 ( $p = .009$ ). A baseline NLR value below 7.45 was positively associated with survival at 180 days (sensitivity 52%, specificity 85.3%,  $p = .025$ ). The presence of substage *b*, was associated with early progression and decreased survival at 180 days ( $p = .031$ ). Anaemia significantly reduced LSSR at 365 days ( $p = .028$ ). This is the first study evaluating PLR and PNR in canine DLBCL and demonstrates that PNR could be a predictor of early lymphoma progression. Since peripheral blood cell composition can be affected by several non-oncological causes, the development of larger multicenter studies with homogeneous inclusion criteria could help to better determine the true predictive values of blood cell ratios in dogs' DLBCL treated with CHOP chemotherapy.

## KEYWORDS

diffuse-large-B-cell, dog, lymphoma, neutrophil-to-lymphocyte, platelet-to-neutrophil, prognosis

## 1 | INTRODUCTION

Non-Hodgkin lymphoma (NHL) is the most common haematopoietic neoplasm in dogs and the most common medically treated tumour in veterinary medicine.<sup>1,2</sup> The validation and use of the World Health

Organization (WHO) scheme to classify canine lymphoma strongly contributed to an accurate comparison between different lymphoma subtypes in clinical studies, eliminating previous bias associated with the inclusion of generalist histotypes.<sup>3,4</sup> Diffuse large B-cell lymphoma (DLBCL) is the most common lymphoma histotype affecting dogs,

contributing up to 47.8% of all B-cell lymphomas.<sup>3</sup> Due to the similarities at molecular, diagnostic and clinical level, the importance of canine lymphoma as a comparative model for the human disease has long been recognized.<sup>5</sup> The shared features between canine and human DLBCL (hDLBCL) justify the search for the use of common therapeutic or prognostic markers which may be beneficial for understanding the disease and improve therapy for both dogs and humans.<sup>1,6,7</sup> The most widely used therapy for canine diffuse large B-cell lymphoma (cDLBCL) consists of a multidrug combination of Cyclophosphamide, Doxorubicin, Vincristine and Prednisolone (CHOP), without maintenance therapy.<sup>2,8,9</sup>

When dealing with cancer, whatever its nature, prognostication is vital. In a clinical environment bench-to-bedside predictors of outcome and response to therapy that are easily accessible, low cost and technically simple to interpret are extremely valuable. Several prognostic factors have been described for canine lymphomas based on patient and tumour intrinsic characteristics, treatment response and host-tumour biological interaction, although the majority of these are based on unspecified lymphoma subtypes and different clinical approaches.<sup>10-14</sup>

In the particular case of veterinary medicine, pre-treatment and easily accessible prognostic information is a very important subject. This data can impact veterinary-client communication, client understanding of prognosis and consequent decision to treat, financial investment and ethical choices.<sup>15,16</sup>

The predictive value of a given prognostic marker may, also, change over time. This means that a prognostic characteristic may differentiate accurately between high-risk and low-risk patients shortly after baseline, but cannot retain this differentiation for a longer period, and thus no longer being valid for clinical decision making.<sup>17</sup>

CHOP based chemotherapy is the standard treatment for canine DLBCL and several variations have been published in recent years with different outcomes, namely with the addition of immune or targeted therapies.<sup>4,18,19</sup>

Inflammation plays a fundamental role in lymphomagenesis and tumour progression.<sup>20,21</sup> Complete blood count is a minimal invasive procedure, inexpensive and is routinely performed in clinical patient's assessment. The study of immune cell subsets from peripheral blood can offer an indication of inflammation and host-tumour interaction.<sup>22</sup> White blood cell indicators, such as absolute monocyte counts<sup>23</sup> and ratios of lymphocyte-to-monocyte (LMR),<sup>24</sup> neutrophil-to-lymphocyte (NLR),<sup>25</sup> platelet-to-lymphocyte (PLR),<sup>26</sup> either individually or in combination,<sup>27</sup> have been studied as prognostic markers in human DLBCL. In the veterinary field, elevated total neutrophil and monocyte counts are associated with a worse prognosis in dogs treated for multicentric lymphoma with a CHOP-based protocol.<sup>28,29</sup> More recently, specific studies found a significant correlation between NLR and LMR on time-to-progression (TTP), progression-free-survival (PFS), lymphoma-specific survival (LSS) and overall survival (OS) for cDLBCL when treated with CHOP-based chemotherapy.<sup>30,31</sup>

Platelets are key effectors in immune processes, and their interactions with other circulating blood cells namely with neutrophils, in inflammation are fundamental for many of their functions.<sup>32,33</sup> Low-platelet count, hypercoagulability and hemostatic disorders have been

described in dogs suffering from lymphoma and shown to impact survival.<sup>9,28,34</sup> The PLR has been described as a prognostic predictor in human DLBCL<sup>26</sup> and canine oral tumours. However, in the latter, with no predictive value of outcome.<sup>35</sup> To our knowledge, the PNR and PLR have not previously been assessed as predictors of outcome for canine lymphoma.

## 2 | OBJECTIVES

We aimed to evaluate the pre-treatment prognostic value of hematologic cell ratios (NLR, LMR, PLR and PNR) for TTP and LSS in a group of dogs with DLBCL, in clinical stage III/IV and treated with a 19-week CHOP chemotherapy protocol.

To better contribute for understanding objective temporal prognosis, we evaluated further the impact of the studied variables at secondary end-points, defined as Time-to-progression-rate (TTPR) and Lymphoma-specific-survival-rate (LSSR) limiting time-to-event landmarks at 180 and 365 days.

## 3 | MATERIAL AND METHODS

### 3.1 | Patient selection and clinicopathological data

Medical records of dogs with lymphoma from a Portuguese Oncology Service (Onevet-Hospital Veterinário Berna) between 2010 and 2016 were reviewed. Patient data extracted from medical records included age, gender, neuter status, breed, haematology values at presentation, lymphoma immunohistotype, WHO stage, substage and chemotherapy treatment received. Inclusion criteria: dogs with a confirmed histological diagnosis of DLBCL, with available pre-treatment haematological data, WHO stage III/IV determined by full clinical examination, thoracic radiographs, abdominal ultrasonography, liver and spleen cytology and peripheral blood smear evaluation and treated with a 19 week CHOP chemotherapy protocol. Patients were excluded if they had received any previous chemotherapy or corticosteroids in the previous month or had stage I/II or V disease. Patients with stage V disease were defined as dogs presenting with circulating neoplastic lymphoma cells, identified on blood smear examination.<sup>30,36</sup>

Chemotherapy consisted of a 19 week CHOP protocol comprising four cycles with a no-treatment week interval between a cycle of a combination of Vincristine (0.5 mg/m<sup>2</sup> IV week 1 and 3), Cyclophosphamide (250 mg/m<sup>2</sup> orally at week 2), Doxorubicin (30 mg/m<sup>2</sup> IV or 1 mg/kg IV according to dog's body weight  $\geq$  15 kg or  $<$  15 kg, respectively) and Prednisolone (1 mg/kg twice a day and tapering for four weeks). Evaluation of treatment response was made, when possible, weekly during treatment and then monthly thereafter, until relapse or death according to published RECIST guidelines.<sup>37</sup>

When relapse or progressive disease was identified, rescue treatment was offered to the dogs' owners, although, rescue treatments were very heterogeneous, and information regarding rescue

chemotherapy was not obtained from the medical records and not considered for purposes of this study.

Haematology values were obtained from the clinical file for every patient. Reference ranges were calculated as the lowest and highest values reported for each parameter.<sup>36</sup> Anaemia, was defined as Packed Cell Volume (PCV) < 39% or Haemoglobin concentration (Hgb) < 11 g/dL, neutrophilia, as absolute neutrophil count above 11 500/ $\mu$ L, and thrombocytopenia, defined as absolute platelet count below  $117 \times 10^3$ / $\mu$ L.

### 3.2 | Statistics

Data were analysed using SPSS version 25. Sample description was made based on absolute and relative frequencies, in the case of the qualitative variables, and mean and SD or median and interquartile range (IQR), in the case of quantitative variables.

Parameters assessed as prognostic variables included gender, neuter status, clinical stage (III/IV), substage (*a* or *b*), presence of anaemia, presence of neutrophilia, presence of thrombocytopenia and the derived cell ratios: NLR, LMR, PLR and PNR.

End-points were defined as per RECIST criteria<sup>37</sup>: TTP as the time between treatment initiation and progression of lymphoma and LSS as the time from treatment initiation to lymphoma related death. Kaplan–Meier curves were used to access both end-points.

Secondary end-points were further evaluated by defining time-to-event intervals, limiting time-to-event (progression or survival) at 180 and 365 days (TTPR180/365 and LSSR180/365).

Cox proportional hazards models (univariate and multivariable models) were used to explore the prognostic impact of variables on overall TTP and LSS.

For the secondary end-points, TTPR and LSSR, animals that did not observe the event, being alive or not showing disease progression, were censored at 180 or 365 days.

Receiving operating curves (ROC) were obtained for haematological ratios that were statistically significant in the survival models. The cut-off values for the different ratios were achieved using the Youden index (Sensitivity + Specificity - 1). The level of significance of  $\alpha = 0.05$  was considered in all analyses.

## 4 | RESULTS

One hundred and fifty-nine dogs were found in the database, and 49.7% (79/159) had a histological diagnosis of DLBCL. From these, two missed stage information, twelve were lost to follow up, and six had documented stage V disease. Fifty-nine dogs met all the inclusion criteria. By the time of the study all dogs have died from their lymphoma. The dogs' characteristics for the study are summarized in Table 1. Eighteen pure breeds were represented, Crossbreed dogs (20), Rottweiler (8), Golden Retriever (4), Labrador Retriever, West Highland Terrier and Cocker Spaniel (3 each), Yorkshire Terrier, Great Dane, Serra da Estrela, Pitt Bull,

**TABLE 1** Patient characteristics of the studied dog population with DLBCL

Characteristics	Numbers
Age (years)	
Median (range)	9.03 (1-17)
Gender n (%)	
Male	28 (47.5)
Female	31 (52.5)
Neutered	25 (42.4)
<b>Tumour characteristics N (%)</b>	
WHO Stage	
III	21 (35.6)
IV	38 (64.4)
WHO substage	
<i>a</i>	33 (55.9)
<i>b</i>	26 (44.1)
<b>Breeds N (%)</b>	
Crossbreed	20 (33.8)
Rottweiler	8 (13.6)
Golden Retriever	4 (6.8)
Labrador Retriever	3 (5.1)
West Highland Terrier	
Cocker Spaniel	
Yorkshire Terrier	2 (3.4)
Great Dane	
Serra da Estrela	
Pitt Bull	
American Staffordshire	
Sharpei	1 (1.7)
Doberman	
Basset-Hound	
Beagle	
Rabbit Hound	
Lhasa Apso	
Bull Terrier	
Irish setter	

American Staffordshire (2 each) and Sharpei, Doberman, Basset-Hound, Beagle, Rabbit Hound, Lhasa Apso, Bull Terrier and Irish setter (1 each).

Regarding treatment, the overall response rate was 86.4% with complete remission in 71.2% (42/59) and partial remission in 15.2% (9/59) of treated dogs. Progressive Disease was identified in 13.6% (8/59) of the patients.

Anaemia was present in 57.6% (34/59) of dogs, 28.8% (17/59) presented neutrophilia and 22% (13/59) were thrombocytopenic. A summary of the dog's haematological data is shown in Table 2.

Table 3 represents data from Cox proportional analysis for TTP and LSS.

Variable	Average (SD)	Median (IQR)	n (%)
NLR	6.52 (4.60)	5.27 (4.15)	
LMR	6.50 (8.24)	4.25 (3.11)	
PNR	0.037 (0.059)	0.026 (0.029)	
PLR	0.17 (0.16)	0.14 (0.17)	
Anaemia PCV (<39%)			34 (57.6)
Anaemia (Hgb <11 g/dL)			19 (32.2)
Neutrophilia			17 (28.8)
Thrombocytopenia			13 (22.0)

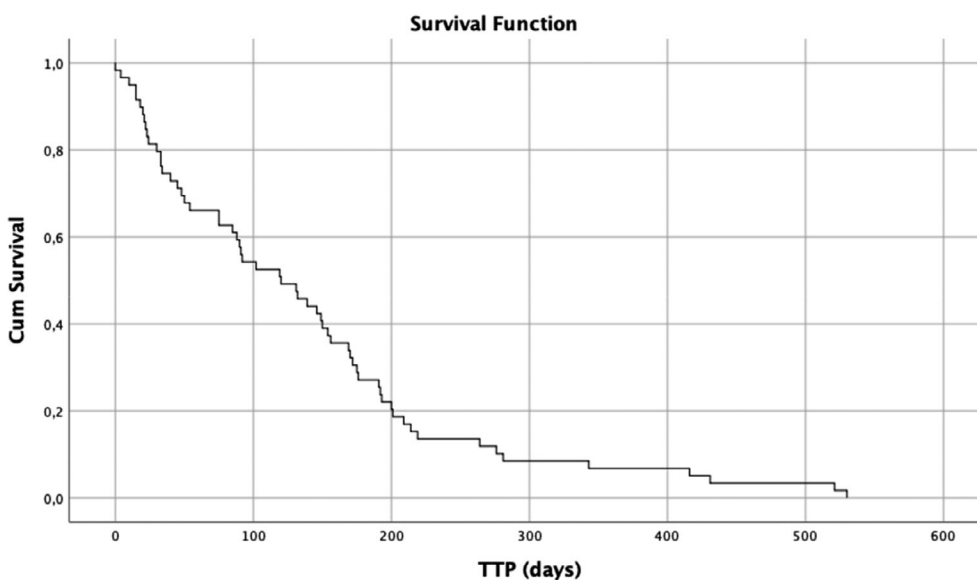
**TABLE 2** Hematologic parameters for the studied population

Abbreviations: SD, Standard Deviation; IQR, Inter-quartile-range; Hgb, hemoglobin; n, number; LMR, lymphocyte-to-monocyte ratio; NLR, neutrophil-to-lymphocyte ratio; PCV, packed cell volume; PLR, platelet-to-lymphocyte ratio; PNR, platelet-to-neutrophil ratio.

**TABLE 3** Cox proportional analysis for the studied variables to time-to-progression and lymphoma-specific-survival

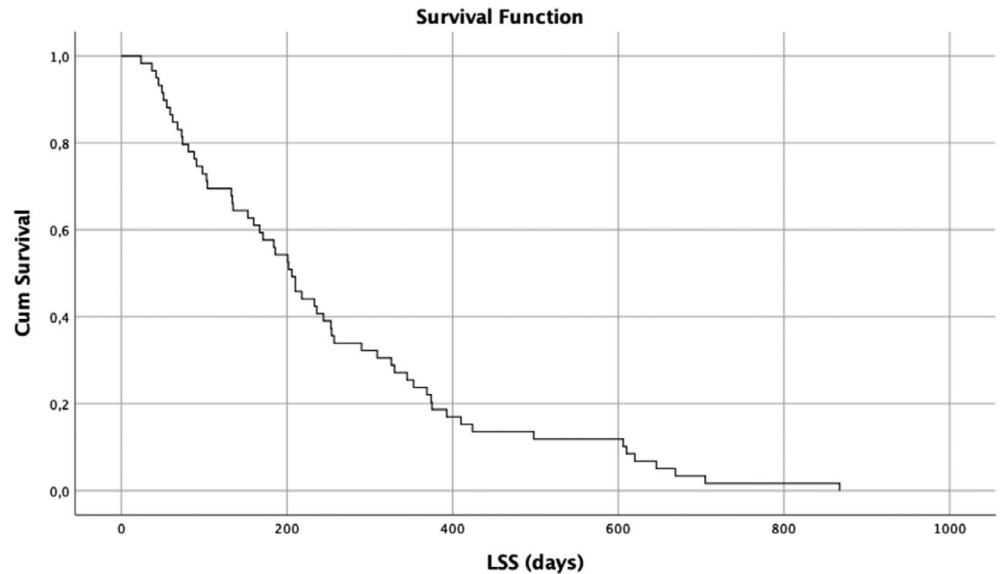
TIME-TO-PROGRESSION	Univariate			LYMPHOMA-SPECIFIC-SURVIVAL			Multivariate		
	HR	CI 95%	P	HR	CI 95%	P	HR	CI 95%	P
	NLR	1.049	0.983-1.120	.148	<b>1.097</b>	<b>1.034-1.164</b>	<b>.002</b>	1.061	0.982-1.146
LMR	1.016	0.982-1.052	.357	0.999	0.960-1.040	.973			
PNR	<b>3.325</b>	<b>1.568-7.050</b>	<b>.002</b>	0.605	0.338-1.083	.091			
PLR	5.790	0.954-35.13	.056	<b>9.536</b>	<b>1.472-61.755</b>	<b>.018</b>	2.675	0.198-24.02	.380
Anaemia PCV < 39%	1.018	0.597-1.736	.947	1.003	0.591-1.704	.991			
Anaemia [Hgb] < 11 g/dL	1.119	0.640-1.957	.694	<b>1.845</b>	<b>1.051-3.239</b>	<b>.033</b>	1.502	0.818-2.756	.189
Neutrophilia	1.070	0.603-1.897	.817	1.550	0.861-2.793	.144			
Thrombocytopenia	1.037	0.556-1.933	.910	1.006	0.569-1.996	.843			
Clinical Stage	1.219	0.700-2.123	.485	1.289	0.741-2.243	.368			
Substage b	1.589	0.939-2.689	.084	1.157	0.679-1.973	.591			
Gender	1.451	0.860-2.449	.163	1.203	0.715-2.026	.486			
Neuter status	0.760	0.449-1.285	.306	0.737	0.432-1.256	.262			

Abbreviations: LMR, lymphocyte-to-monocyte ratio; NLR, neutrophil-to-lymphocyte ratio; PCV, packed cell volume; Hgb, hemoglobin; HR, Hazaed-ratio; CI, Confidence-interval; PLR, platelet-to-lymphocyte ratio; PNR, platelet-to-neutrophil ratio; p value is in the cell at the right of each value also in bold.



**FIGURE 1** Kaplan-Meier curve representing time-to-progression (TTP) of the study population, Median overall TTP was 120 days (95% CI- 68.4-171.6)

**FIGURE 2** Kaplan–Meier curve representing overall lymphoma-specific-survival (LSS). Median LSS was 206 days (95% CI 169.4–242.6)



#### 4.1 | Progression

Median overall TTP was 120 days (CI95%, 68.38–171.61), represented in Figure 1.

On univariate analysis only Platelet-to-neutrophil ratio (HR = 3.325;  $p = .002$ ) revealed significance for TTP as PNR increases, more likely the animal will suffer from lymphoma progression.

Platelet-to-neutrophil ratio showed an independent prognostic factor for lymphoma progression at 180 days ( $p \leq .001$ ). Presence of substage *b* ( $p = .031$ ) revealed significance, only at univariate analysis for TTPR180.

Dogs with a PNR above 0.032, depicted at Figure 3, have higher risk of disease progression before 180 days (sensitivity 46.5%, specificity 87.5%,  $p = .044$ ).

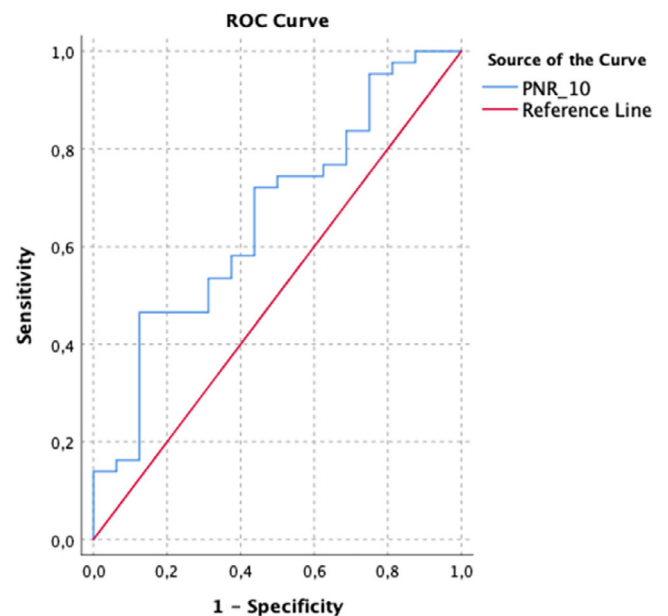
#### 4.2 | Survival

The lymphoma specific median survival time was 206 days (CI95%, 169.44–242.56) and the percentage of animals alive at 180 and 365 days, was 57.6% (34/59) and 23.7% (14/59), respectively (Figure 2).

On univariate analysis, NLR (HR = 1.097,  $p = .002$ ), PLR (HR = 9.536,  $P = .018$ ) and anaemia (Hgb < 11) (HR = 1.845,  $p = .033$ ) showed to have significant impact for overall survival (Table 3).

NLR ( $P = .006$ ) and presence of substage *b* ( $P = .031$ ) showed a prognostic significance for LSSR at 180 days (Table 4). Dogs with NLR value  $\geq 7.45$  (sensitivity 52%, specificity 85.3%,  $p = 0.025$ ) were most likely to die of lymphoma before 180 days (Table 5).

Significant prognostic factors for LSSR at 365 days were NLR ( $p = .009$ ) and anaemia (haemoglobin < 11 g/dL) ( $P = .028$ ). But both variables lost its significance in the multivariate model (Table 4).



**FIGURE 3** Receiving operating curves (ROC) curve analyses for platelet to neutrophil ratio cut-off determination for the time-to-progression-rate (TTPR) at 180 days (AUC = 0.660, Sensitivity = 0.465, specificity = 0.875,  $P = .044$ )

## 5 | DISCUSSION

Numerous prognostic factors have been studied and evaluated for dogs with multicentric lymphoma,<sup>11,14</sup> although for DLBCL there are very limited reports at present.<sup>28,30,31</sup>

For the first time, in this study we evaluated and described the impact on prognosis of the PLR and PNR in dogs with DLBCL.

Animals presenting a PNR value above 0.032 (AUC = 0.660,  $P = .004$ ) tend to have earlier disease progression. PNR is a direct

**TABLE 4** Cox Proportional analysis of variables affecting rates of TTP and LSS at specific time landmarks (180 and 365 days)

Factor	Univariate						180 days
	TTPR			LSSR			
	HR	CI95%	p	HR	CI95%	p	
NLR	1.053	0.984-1.127	.134	<b>1.099</b>	<b>1.027-1.175</b>	<b>.006</b>	
LMR	1.023	0.986-1.062	.220	1.040	0.999-1.083	.058	
PNR	<b>3.355</b>	<b>1.553-7.250</b>	<b>.002</b>	0.302	0.044-2.099	.226	
PLR	6.641	0.951-46.36	.056	7.996	0.730-87.57	.089	
Anaemia PCV <39%	1.379	0.747-2.547	.304	1.430	0.632-3.238	.391	
Anaemia Hgb<11g/dL)	1.347	0.718-2.526	.353	2.029	0.920-4.476	.079	
Neutrophilia	0.924	0.474-1.800	.816	1.283	0.553-2.974	.561	
Thrombocytopenia	1.053	0.504-2.199	.890	1.712	0.715-4.100	.228	
Clinical Stage	1.276	0.674-2.418	.454	1.199	0.517-2.779	.673	
Substage	<b>1.944</b>	<b>1.064-3.552</b>	<b>.031</b>	<b>2.412</b>	<b>1.082-5.377</b>	<b>.031</b>	
Gender	1.593	0.870-2.916	.131	1.504	0.682-3.316	.311	
Neutering	0.762	0.413-1.407	.385	0.706	0.312-1.598	.403	
Factor	Multivariate						180 days
	TTPR			LSSR			
	HR	CI95%	P	HR	CI95%	P	
PNR	<b>4.969</b>	<b>2.084-11.85</b>	<b>&lt;.001</b>				
Substage	1.818	0.983-3.360	.057	1.741	0.749-4.403	.197	
NLR				1.052	0.987-1.121	.121	
Factor	Univariate						365 days
	TTPR			LSSR			
	HR	CI95%	P	HR	CI95%	P	
NLR	1.049	0.983-1.120	.149	<b>1.087</b>	<b>1.021-1.158</b>	<b>.009</b>	
LMR	1.016	0.981-1.052	.373	1.015	0.974-1.057	.478	
PNR	<b>3.340</b>	<b>1.571-7.100</b>	<b>.002</b>	0.641	0.286-1.437	.280	
PLR	5.770	0.945-35.25	.058	6.652	0.915-48.36	.061	
Anaemia PCV <39%	1.078	0.629-1.847	.786	1.169	0.646-2.113	.606	
Anaemia (Hgb<11g/dL)	1.234	0.701-2.172	.466	<b>1.983</b>	<b>1.078-3.647</b>	<b>.028</b>	
Neutrophilia	1.070	0.603-1.897	.817	1.435	0.769-2.677	.256	
Thrombocytopenia	0.993	0.522-1.891	.984	1.285	0.636-2.597	.484	
Clinical Stage	1.116	0.639-1.950	.700	1.028	0.558-1.893	.930	
Substage	1.481	0.865-2.535	.152	1.459	0.808-2.633	.210	
Gender	1.409	0.823-2.412	0.211	1.057	0.588-1.901	0.852	
Neutering	0.787	0.460-1.349	0.384	0.816	0.449-1.483	0.505	
Factor	Multivariate						365 days
	TTPR			LSSR			
	HR	CI95%	P	HR	CI95%	P	
Anaemia Hgb				1.494	0.765-2.916	.240	
NLR				1.053	0.987-1.123	.119	

Abbreviations: CI, confidence interval; HR, hazard ratio; Hgb, haemoglobin; LMR, lymphocyte-to-monocyte ratio; LSSR, lymphoma-specific-survival rate; NLR, neutrophil-to-lymphocyte ratio; PCV, packet cell volume; PLR, platelet-to-lymphocyte ratio; PNR, platelet-to-neutrophil ratio; TTPR, time-to-progression rate; p value is in the cell at the right of each value also in bold.

**TABLE 5** Cut-off values determined by ROC analysis for the significant variables for the rates of progression and survival at 180 and 365 days

Ratio	180 days					365 days				
	TTPR									
	Cut-off	<i>P</i>	AUC	Sens.	Sp.	Cut-off	<i>P</i>	AUC	Sens.	Sp.
PNR	<b>0.32</b>	<b>0.044</b>	0.660	0.465	0.875	0.32	0.203	0.636	0.400	1.000

Ratio	180 days					365 days				
	LSSR									
	Cut-off	<i>P</i>	AUC	Sens.	Sp.	Cut-off	<i>P</i>	AUC	Sens.	Sp.
NLR	<b>7.45</b>	<b>0.025</b>	0.664	0.52	0.853	6.44	0.105	0.625	0.467	0.929

Abbreviations: AUC, Area-under-the-curve; Sens., Sensitivity; Sp., Specificity; LSSR, lymphoma-specific-survival-rate; NLR, neutrophil-to-lymphocyte ratio; PNR, platelet-to-neutrophil ratio; ROC, receiving operating curves; TTPR, time-to-progression-rate; The significance values are in the cell immediately at right of each bold value.

result of the interplay between platelets and neutrophils in malignant conditions, especially when hemostatic abnormalities are present, such as malignant hypercoagulability, a frequent paraneoplastic syndrome in humans<sup>38</sup> and dogs with lymphoma.<sup>34,39</sup> Neutrophils promote inflammation via the inflammasome pathway and neutrophil extracellular traps, contributing to tumour progression.<sup>33</sup> Furthermore, tumour associated neutrophils can protect tumour cells against cytotoxic therapy by CD11b/ICAM-1 binding, reducing lymphoma cell susceptibility to therapy and facilitating early progression.<sup>40</sup> The presence of several coagulation mediators in hypercoagulability syndrome plays a fundamental role in perpetuating inflammation and consequently, cancer progression. One of these coagulation components - Tissue-Factor - has been previously described to be present and associated with early relapse in human lymphoma,<sup>41,42</sup> and we cannot rule out the same effect in cDLBCL, contributing also for lymphoma progression.

Dogs with NLR value below 7.45 at presentation (Table 5) are more likely to be alive at 180 days (sensitivity 52%, specificity 85.3%, *P* = .025). The NLR has been previously reported as a predictor of longer PFS and OS<sup>30</sup> in univariate analysis for cDLBCL but was not significant when evaluated for a mixed lymphoma population.<sup>43</sup> To date, there are no reports of the persistence of its independent predictive value in multivariate analysis. In humans with DLBCL, higher values of NLR are associated with poorer outcomes in both PFS and OS.<sup>43</sup> Higher ratios can be due to an increased number of neutrophils, that may lead to immunosuppression and tumour progression by decreased suppression of cytolytic action of lymphocytes, natural killer and CD8 cells, production of nitric oxide synthase down-regulating the antitumor activity of lymphocytes contributing to angiogenesis and tumour proliferation and progression.<sup>25,44</sup> Care must be taken when interpreting NLR since, in humans, several demographic and lifestyle factors can affect NLR<sup>45</sup> values and dogs may be affected by the same or similar factors.

We did not find a significant association between LMR and cDLBCL progression and survival, despite LMR being close to significance (*P* = .058) for LSSR at 180 days (Table 4). Higher values of LMR have been previously associated with longer OS in cDLBCL,<sup>30,31</sup> or

prolonged PFS for dogs with mast cell tumours.<sup>46</sup> In human oncology, LMR has been inconsistent in predicting prognosis, but lower values are associated with poorer outcomes in hDLBCL.<sup>47</sup>

High values of PLR in hDLBCL is associated with shorter OS and PFS,<sup>48</sup> but for dogs, to the authors' knowledge, the only report evaluating PLR in veterinary medicine describes a higher PLR value in dogs with oral tumours when compared to healthy dogs and dogs with periodontal disease.<sup>35</sup> In our study, close but not statistically significant values were obtained for PLR impacting TTPR at 180 days (*P* = .056) and 365 days (*P* = .058). In hDLBCL high PLR is associated with poor OS<sup>26</sup> and, despite our results, a further evaluation of the prognostic impact of PLR in cDLBCL is justified.

The presence of anaemia has been previously identified as a prognostic indicator for dogs treated with CHOP-based chemotherapy<sup>49</sup> but not confirmed by others.<sup>9,50</sup> In our study, anaemia (as per low haemoglobin concentration) was significantly associated with LSSR at 365 days (*P* = .028). The pathogenesis contributing to anaemia in dogs suffering from lymphoma is undetermined. However, chronic disease with associated bone marrow erythroid hypoplasia, erythrocyte decreased survival, auto-immune hemolytic anaemia, abnormal iron reutilization, decreased erythropoietin production, and high levels of IL6 are possible contributors.<sup>51-53</sup> The possible impact of anaemia on survival may come from the presence of chronic hypoxia leading to the selection of lymphoid cells which are more resistant to treatment.<sup>54</sup> Also, hypoxia associated cytokines in the plasma, such as vascular endothelial growth factor or metalloproteinases may contribute to tumour progression and aggressiveness.<sup>54</sup> Anaemia may also impair the quality of life of dogs causing fatigue, exercise intolerance, worsening of compensated underlying comorbidities (cardiovascular, renal, pancreatic) and behaviour/cognitive impairments, which could lead to treatment reduction or delay. This impairment of quality of life due to anaemia can compromise the outcome, leading to early treatment stop or euthanasia and reducing survival.

Dogs in WHO substage *b*, had significant early lymphoma progression (TTPR) and decreased survival (LSSR) at 180 days (*P* = .031). Shorter progression times in dogs with substage *b* have been

described in previous reports for cDLBCL<sup>31</sup> but not confirmed by others.<sup>30</sup> Despite substage *b* being a frequent indicator of poor outcome in populations of mixed lymphoma histotypes impacting early relapse,<sup>9,49,55</sup> specifically for cDLBCL, its real prognostic value needs more data to support it. One crucial step that needs to be considered, and would contribute significantly for determining its real prognostic value, is to have objective criteria to define substage *b* and not being dependent on subjective clinician considerations.<sup>56</sup>

In people, substage *b* is associated with increased toxicity of treatment, such as the development of severe cytopenias, vomiting and diarrhoea.<sup>57</sup> This increased sensitivity to treatment can be secondary to the presence of high levels of systemic inflammatory markers, such as CRP,<sup>57,58</sup> leading to acute or chronic onsets of comorbidities with treatment implementation, contributing to treatment delays or dose reduction compromising therapeutic efficacy. In dogs, probably the underlying mechanisms are the same, and increased treatment-associated toxicities can also lead to an early decision on euthanasia or treatment change.

The use of secondary end-points, in our study, defined by TTPR and LSSR at defined temporal landmarks was an attempt to provide a temporal prognosis that may help clinicians to communicate prognosis in a possibly more understandable way to clients and, consequently, assist in treatment decisions. RECIST criteria have several limitations, namely regarding the assessment of disease progression.<sup>37</sup> The detection of progression depends of the methodology used, the temporal landmarks defined to assess disease status and inter-observer variations. Secondary end-points, at specific time landmarks, in this case at 180 and 365 days, may be useful to better understand treatment response and associated prognosis and analyse how specific prognostic variables maintain their accuracy over time<sup>17</sup>. Interestingly, in our study, PNR kept its significance when assessed for overall TTP or for the 180 and 365 time landmarks. The same was verified for NLR and anaemia for LSS.

We did not find any association between total neutrophil and platelet counts with disease progression and survival (Table 2). Thrombocytopenic dogs with multicentric lymphoma at presentation were found to have the worst outcome, with decreased progression free interval (PFI) and/or OS.<sup>9,59</sup> In the particular case of cDLBCL, low platelet count was also predictive of partial remission and shorter PFI.<sup>28</sup> Increased neutrophil counts were associated with shorter disease-free-interval and PFS in dogs treated with CHOP with undefined multicentric<sup>29,49</sup> or diffuse large B-cell lymphoma,<sup>28</sup> respectively.

In humans with DLBCL, the presence of thrombocytopenia is associated with poor outcome and is related to the systemic inflammatory status, with upregulation of inflammatory markers, bone marrow invasion and autoimmune destruction.<sup>60,61</sup> In dogs, the mechanisms underlying lymphoma associated thrombocytopenia have not been elucidated, but possibly could be similar to those described for humans.

We could not find any prognostic impact of gender or neutering in DLBCL progression or survival, despite reports of better outcomes in females<sup>10,62</sup> or neutered females.<sup>63</sup> These studies did not

differentiate between histological type and phenotype of lymphoma and treatments were other than CHOP, so it is not possible to make an objective comparison. Specifically for DLBCL, gender was significant for LSS when the treatment was chemoimmunotherapy,<sup>31</sup> but did not keep its prognostic significance for dogs treated with pure CHOP protocols;<sup>28,30</sup> studies are warranted to evaluate the real impact of gender in cDLBCL.

Our study has limitations due to its retrospective nature and relatively small sample size; the latter may have limited statistic power and lead to possibly missing significant associations. In our study, we had 13.6% (8/59) of patients with progressive disease and, from those, 62.5% (5/8) were euthanized around day 28th after completing the first cycle of CHOP without achieving remission (data not shown). This fact can be attributed to the possible presence of primary refractory hard-to-treat lymphoma, potentially influencing survival.<sup>64</sup> Also, the lack of bone marrow evaluation to properly assess stage V, or the lack of use of advanced diagnostic techniques such as PARR or flow cytometry from peripheral blood, could have contributed to downstaging in some dogs, and the inherent risk of dogs in stage V disease being included resulting in misleading results, since peripheral blood abnormalities not always correlate with bone marrow involvement.<sup>65</sup>

Some causes that could have contributed to the difference observed between our study and the previous study evaluating haematological ratios on cDLBCL treated with CHOP may be due to the exclusion of stages I/II and V, loss of accuracy inherent to discrepancies in automatic/manual cell counts and the inclusion of stage V dogs which could not be detected by blood smear evaluation. Additionally, disparities in treatment such as CHOP, L-CHOP and COP<sup>30</sup> or potentiating CHOP therapy with immunotherapy.<sup>31</sup>

Due to the several non-specific pathological, demographic and physiological factors that can promote changes in white blood cell kinetics in peripheral blood, a critical evaluation needs to be performed when assessing haematological derived ratios as prognostic markers.<sup>66</sup>

We described for the first time the use of PNR as a strong predictor of time-to-progression and consolidate NLR, anaemia and substage *b* as prognostic indicators in canine diffuse large B cell lymphoma, treated with CHOP chemotherapy. We also evaluated and described for the first time the PLR as a possible promising prognostic marker in dogs with DLBCL.

Consequently, the true impact of haematological ratios in treatment decision or treatment adaptation (which protocol, doses, frequency and duration) still needs to be evaluated, but this study adds valuable information to future research on personalized medicine and risk stratification. The addition of complementary diagnostic techniques to further subtype white blood cell subpopulations, namely flow cytometry, can add important insights and contribute to define new and/or increase information on specific ratios.

The validation of haematological ratios as significant prognostic markers in veterinary oncology still needs to undergo further and vast research, with the inclusion of larger populations of dogs, assessed by comparable multicentric studies and further respective

meta-analysis to determine clinical relevance, as is the case in the human field.<sup>47,67</sup>

This work contributes to the early clinical studies on canine DLBCL, although more studies, with a larger number of patients with consistent inclusion and treatment criteria, are needed to consolidate knowledge on treatment response and prognosis in cDLBCL treated with CHOP chemotherapy.

#### DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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**7. Comment on “Being rational over blood ratios in veterinary hematology”**

# Comment on “Being rational over blood ratios in veterinary hematology”

We are thankful to Simões and Marcos for their letter to the editor following our recent work.<sup>1</sup> The letter raises very pertinent and interesting arguments, complementing the published literature on the subject and contextualizes a rationale approach on blood cell ratios (BCR).<sup>2</sup>

We want to address our reply as comments to the main topics approached in the letter:

**Blood smear evaluation and pitfalls of automatic analyzers:** Manual differential count, despite being the gold standard method, can also be affected by inter-observer variation and by the lower number of cells evaluated, namely, when leucopenia exists.<sup>3</sup> If BCR becomes widely used by clinicians and calculated upon manual differentials, it will demand human and time-consuming resources especially, if normoblastemia and leukopenia are relevant and complex calculations for leucocyte corrections are needed.<sup>4</sup> In this way, test cost and time delay in reporting, can, possibly, limit BCR principal advantages and clinical applicability.

Despite the few limitations described of veterinary automatic analyzers in detecting and allocate all the blood cell populations accurately,<sup>3,5</sup> it is expected that soon these limitations will disappear or become significantly reduced, with the availability of more advanced equipment and combined software for the veterinary field. A significant remark is that Sysmex or Advia equipment is usually located at referral labs or teaching institutions, where automatic reports are critically analysed by specialized and experienced staff, contributing largely to decrease the aforementioned pitfalls.

**Nucleated red blood cells as another low-cost prognostic factor in lymphoma:** There might be a promising value, yes. Nevertheless, lymphoma-associated anaemia is, usually, non-regenerative<sup>6</sup> and normoblastemia should be rationally contextualized with undergoing non-anaemic-associated mechanisms such as high levels of circulation erythropoietin,<sup>7</sup> levels of circulating cytokines (such as vascular endothelial growth factor<sup>8</sup>), among others, that may contribute to bone marrow endothelial vasculature permeability.

In conclusion, it is important to stress that the predictive value of a given prognostic marker may change over time, impacting accurate patient risk stratification and unsatisfactory clinical applicability, no longer being valid for clinical decision making.<sup>9,10</sup> This can create difficulty for clinicians to accurately apply and integrate this information into practice, without validated algorithmic approaches.<sup>11</sup>

The validation of haematological ratios, nRBC or other prognostic markers in veterinary oncology needs to undergo further and extensive research delineated by strict guidelines<sup>12</sup> and prognostic investigation frameworks to determine the real clinical relevance, supported

by the development of prognostic algorithms approaches as in the human field.<sup>13</sup>

We are all making efforts to go in the right direction!

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## 8. Final discussion

The present work analyses and describes, for the first time in Portugal and in Europe, the association between vector-borne agents and lymphoma in dogs. Secondly, it was also evaluated, for the first time, new possible peripheral blood cell ratios (platelet-to-neutrophil ratio and platelet-to-lymphocyte ratio) as prognostic markers in dogs with DLBCL.

Despite the several reports on the association of CVBD and lymphoma in dogs<sup>36,37,135</sup> and humans,<sup>39,506</sup> only one study was performed to assess the presence of *Bartonella spp.* in Golden Retrievers with lymphoma and healthy controls.<sup>35</sup> Literature is lacking on this subject. Here it was demonstrated that dogs with lymphoma, living in the metropolitan area of Lisbon, have a non-significant increase in infection for *Leishmania infantum*, and are seronegative and show no molecular evidence of *Bartonella henselae*, *Ehrlichia canis* and *Anaplasma phagocytophilum* infection, when compared to controls from the same geographic region.<sup>38, 508,512</sup>

*Leishmania infantum* detection in lymphoma patients vs. historic controls by serology (8,2% vs. 7,9%) and molecular (3,3% vs. 1,2%) methods was not significantly different, ( $p=0,201$  and  $p=0,166$ ), respectively.

Although previously described,<sup>513</sup> in the present work the presence of co-infections of *Leishmania infantum* with the other studied vector-borne agents, was not detected. Also, serologic and molecular detection for *E. canis* and *A. phagocytophilum* is described in domestic (in-house) dogs in the Lisbon area, but no evidence of these agents was detected in the study population of dogs with lymphoma.<sup>508,512</sup> This discrepancy can be due to the small number of dogs enrolled or the effective use of ectoparasiticide by dog's owners eliminating or decreasing vector exposure. The negative results for the detection of *Bartonella henselae* comes in line with the fact that, to the authors knowledge, no evidence of Bartonella genus microorganisms has been described in Portugal.<sup>512</sup>

The fact that only *Leishmania infantum* was detected in dogs with lymphoma can be due to several causes: the vaccine for Leishmaniosis was only available in Portugal after May 2011<sup>514</sup> and had an high associated cost when compared to the other vaccines, potentially compromising owner compliance and, consequently, increasing risk of infection. Other factor that may have contributed to increased the risk of *Leishmania infantum* organisms infection relates to failure on the implementation of effective preventive chronic use of synthetic pyrethroids.<sup>515</sup> This preventive measure can be also associated with significant economic cost, compromising client compliance and increasing the risk to sand flies bites and infection. All the dogs were diagnosed with leishmaniosis before developed their lymphomas. The reason to referral was the cancer and for deontological reasons the referring vets kept the

medical management of the leishmaniosis. Treatments of seropositive dogs were very variable in molecules, doses and therapeutic schemes, reasons that justified the decision not to have a clinical record of the dogs during the course of their leishmaniosis.

In humans, visceral leishmaniosis has been mainly associated with different entities of lymphoma<sup>512</sup> and in dogs with T-cell immunophenotype.<sup>36,37</sup> In human oncology it has been raised the cancer promoting potential of *Leishmania spp.* infection, namely lymphoma.<sup>34, 511,516</sup>

Dogs affected by *Leishmania spp.*, studied and clinically followed during the course of their disease can be a good natural model to study the mechanisms by which *Leishmania* organisms can promote lymphomagenesis. Those mechanisms already described, comprise chronic inflammation, immune suppression and senescence, epigenetic events, depletion of Th1 response and increase Th2 cytokine response.<sup>29,133,134</sup>

Despite the low numbers of seropositive dogs for *L. infantum* (8,2%), it is interesting to notice that 60% suffered from DLBCL. The remaining 40% developed indolent forms (Mantle and Small cell lymphoma). The two dogs with molecular evidence of *L. infantum* were also affected by DLBCL. Due to the low number of DLBCL cases (n=37) in this study, it was not possible to deduct any plausible association between DLBCL and *L. infantum* positive cases. Unfortunately, the funding for this work ended before the accomplishment of a significant number of dogs to extend the analyses to a larger population, increasing the statistic power and possibly obtain different results. It is possible that in dogs as in humans, *Leishmania spp.* can co-exist in different entities of lymphoma, but its true importance in lymphomagenesis has still to be proven.

Dogs infected with *Leishmania spp.*, *Ehrlichia spp.*, *Anaplasma spp.* and *Bartonella spp.* can have a wide spectrum of associated clinical and laboratorial abnormalities. These include lymphadenomegaly or hepatosplenic, uveitis, immune-mediated diseases, anaemia, leucocyte and platelet abnormalities and gastrointestinal symptoms, among others.<sup>509,510,517</sup> This justifies the reason for the evaluation of the prevalence of those infectious agents before the study on peripheral blood cells ratios and prognosis. These diseases can contribute strongly to worse prognosis in dogs affected by lymphoma by several ways: 1) owners could be more resistant to treat dogs with lymphoma if they have a VBD, since they already have spent emotional, time and financial resources to treat the pre-existent disease; 2) the severity and number of clinicopathological abnormalities due to the VBD can mimic lymphoma progression or limit treatment choice and administration; 3) haematological complications such as non-regenerative anaemia, thrombocytopenia or leukopenia; 4) azotaemia due to impaired renal function; 5) hepatosplenomegaly; 6) lymphadenomegaly and 7) immune mediated disorders (polyarthritis, anaemia, glomerulonephritis, etc.) are some of the

examples of these clinicopathological events. The presence of one or more of the aforementioned complications contribute to determine prognosis by increasing susceptibility to treatment, increasing toxicity or falsely influence staging, either by the presence of reactive lymphadenopathy and /or hepatic-splenomegaly or lead to wrong classify as substage b due to the presence of similar symptoms, namely gastrointestinal.<sup>40,470</sup>

In the United Kingdom, first opinion practices treat at least 50% of the lymphoma cases they diagnose.<sup>518</sup> In Portugal, cytotoxic chemotherapy is widely and easily available in veterinary medicine and, in the author's experience, lymphoma is diagnosed and treated mostly in first opinion practices, leaving the referral for cases of relapsed, advanced stage, dogs with multiple co-morbidities or by request of the owner to seek referral veterinary services. These factors have contributed to increase the time of the inclusion period and reduced the number of dogs fulfilling inclusion criteria in the study. Another case limiting situation was that during the period of recruitment of cases, the mainstream diagnostic approach to canine lymphoma was cytology.<sup>344</sup> The idea to perform even a brief anaesthesia to obtain a tumour biopsy to diagnose and classify the lymphoma according to the WHO entity, based on morphologic and IHC, was not largely accepted by owners or referral veterinarians.

Also, the low percentage of concomitant lymphoma and leishmaniosis in dogs in the same individual, can attribute it to bias. Nevertheless, a prospective study, including a significant number of dogs infected by *Leishmania spp.*, following the course of their disease can help understand a putative *Leishmania spp.* driven lymphomagenesis,<sup>511</sup> or, inversely, the lymphoma associated increased risk to be infected by this intracellular parasite.

From the five dogs that were positive for *Leishmania infantum*, only one, suffering from B small cell lymphoma, did not show any hematologic abnormality. Although the antibody titre was high (1:2560), there was no molecular evidence of *Leishmania infantum* in the lymphnode. All the other dogs, presented haematological abnormalities, but none considered severe. Mild anaemia was present in two dogs, one of these with concurrent mild thrombocytopenia. Moderate monocytopenia and lymphocytopenia were present in one dog each. All the peripheral blood alterations seen in these dogs were mild and not associated with lymphoma type, serologic titre or molecular positivity. It was interesting to notice, that dogs with the same high antibody titres (1:2560) had different associated haematological changes: one only mild monocytopenia, one mild anaemia and thrombocytopenia and other no abnormalities, despite the contrary being described.<sup>519</sup> The types of lymphoma were also different and the dog without clinical abnormalities suffered from B small cell lymphoma. It was not possible to differentiate if these hematologic abnormalities are related with the lymphoma and/or the Leishmaniosis, but probably those changes are due to the action of both diseases in bone marrow.<sup>258,267,509</sup>

These findings support the initial hypothesis that concomitant vector-borne infections interfere with peripheral blood population kinetics, compromising the results and applicability of peripheral blood cell and their ratios as prognostic factors for canine lymphoma.

Dogs affected by Leishmaniosis, when compared with the studied population, had shorter median TTP and LSS, 90 vs. 120 and 184 vs. 206 days, respectively. Due to the very small number of VBD positive dogs, it was not possible to take any significant conclusion on the association between the studied CVBD either with the specific lymphoma types or the impact in peripheral blood cell anomalies. Nevertheless, this first study is relevant as, it evaluates for the first time in Portugal and Europe, the potential association of CVBD and lymphoma and allowed us to have ground base information, to be discussed in the second publication.

The prognostic value of peripheral blood cells in cL and their derived ratios has received in the last decade attention from the veterinary scientific community.<sup>227,498,505</sup> The present work evaluated and described, for the first time, pre-treatment platelet-to-neutrophil ratio and platelet-to-lymphocyte ratio as prognostic markers in dogs with DLBCL.<sup>497</sup> PNR demonstrated to be an independent prognostic marker for time-to-progression progression rate at 180 and 365 days ( $p < 0,001$ ). A PNR above 0,032 (sensitivity 46,5%, specificity 87,5%,  $p = 0,004$ ) is associated with increased rate of progression at 180 days, in dogs with DLBCL treated with CHOP protocol. In the present study close but not significant association was found between PLR and DLBCL overall TTP ( $p = 0,0056$ ) and rate of progression at 180 ( $p = 0,056$ ) and 365 days ( $p = 0,058$ ). Also, the PLR showed to be indicator of overall survival ( $p = 0,018$ ) as already described in humans.<sup>520</sup> NLR ( $p = 0,006$ ) and presence of substage *b* ( $p = 0,031$ ) correlated with Lymphoma-Specific-Survival rate (LSSR) at 180 days. Dogs with NLR value  $\geq 7,45$  (sensitivity 52%, specificity 85,3%,  $p = 0,025$ ) were most likely to die of lymphoma before 180 days. Significant prognostic factors for LSSR at 365 days were NLR ( $p = 0,009$ ) and anaemia (haemoglobin  $< 11$  g/dL) ( $p = 0,028$ ). But both variables lost its significance in the multivariate model. Substage *b*<sup>12,468,158,346,497</sup> and anaemia<sup>253,347,253,347,28,302</sup> are consolidated negative prognostic factors and were reconfirmed in the present study for dogs in stage III/IV treated with 19 week CHOP protocol. Mild anaemia was present in 40% (2/5) of the dogs infected with *Leishmania infantum*, possibly due to the association of the tumour and the underlying chronic infectious disease. Thrombocytopenia, lymphocytopenia and monocytopenia were each one present in 20% (1/5) of dogs with leishmaniosis. The possible impact of these specific cytopenias in influencing their derived ratios, namely NLR, LMR and PLR is yet to be demonstrated. These results keep suggesting a possible negative impact of CVBD in peripheral blood cell and consequently, affecting prognostic

determination. A future study, with a larger population of dogs followed in the course of their disease is needed to validate this suspicion.

The determination of the blood cell values was made with validated automatic analysers for veterinary medicine. Several limitations have been identified with automatic cell analysers that can interfere with automatic blood cell counts and respective derived ratios, namely in detecting and allocate all the blood cell populations accurately.<sup>521</sup> Manual confirmation of all the counts increased the sensibility and sensitivity of the results outputted by the analysers. It is expected that soon these limitations will disappear or become significantly reduced, with the availability of more advanced equipment and combined software for the veterinary field.<sup>522</sup> Nevertheless, it is always recommended to validate the automatic output of the analysers with a manual visual count to determine the possible impact of wrong cell count or allocation. This is particularly important when normoblasts are present, either due to regenerative anaemia or as paraneoplastic syndrome.<sup>523</sup> Ways to overcome these limitations and the positioning of peripheral blood cell ratios in veterinary oncology were largely discussed in the third publication.<sup>522</sup>

Comparative oncology has gained an increased interest and expansion in the latest years, namely under the umbrella of the WHO One Health initiative. The possibility of understanding shared cancer-underlying mechanisms at molecular, cellular, immunologic, clinical and therapeutic fields in both humans and animals opens a door to a brighter future where both human and veterinary patients can strongly benefit. Companion animals with natural occurring diseases, sharing the same risk factors than their household humans, provide an amazing possibility as translational models in human oncology.<sup>20,22</sup>

The present work enabled the validation of new prognostic factors for canine diffuse large B cell lymphoma and contributed also to understand the underlying mechanisms and concomitant diseases that can either affect blood cell pool and /or mimic lymphoma or substage b and, consequently, impact therapeutic decisions and prognosis.

## 9. Conclusions

Having in consideration the results of the published works, either individually and the contextualization in general discussion, the present work has fulfilled the proposed objectives.

Dogs with lymphoma demonstrated negative antibodies and molecular detection for the canine vector-borne agents *Ehrlichia canis*, *Anaplasma phagocytophilum* and *Bartonella henselae*. *Leishmania infantum* seropositivity and molecular detection was similar to the general population in the same geographic area. *Leishmania infantum* seropositivity and molecular detection was associated with cases of DLBCL. Although it was not possible to detect a higher prevalence of dogs with lymphoma exposed or infected with CVBD, these infectious agents should be part of a lymphoma diagnostic work up, due to the clinical impact in the patients, causing co-morbidities and, consequently conditioning response to therapy and prognosis.

Since co-infection with CVBD in dogs with lymphoma and, specially DLBCL, is very low, it is possible to conclude that the impact on disease prognosis, bone marrow and /or peripheral blood cell pool can be considered to be very low. Nevertheless, dogs with lymphoma, and especially with DLBCL should be tested for *Leishmania spp.*, by means of serologic and/or molecular detection, since this disease can impact prognosis, namely by causing cytopenias.

New prognostic factors based on the pre-treatment blood cell counts and ratios were validated in dogs affected by DLBCL, particularly when assessing disease progression and survival at 6 and 12 months. Anaemia as per low haemoglobin concentration significantly conditions survival rate of dogs at 12 months; platelet-to-neutrophil ratio above 0,032 is associated with early disease progression. The results obtained contributed to consolidate the prognostic importance of the pre-treatment neutrophil-to-lymphocyte ratio and its impact on survival rate at 180 days. Our results did not validate previous described prognostic factor for “multicentric lymphoma” such as sex, neutering status, neutrophilia and thrombocytopenia for cDLBCL.

The state of art methodology used for diagnostic, staging, lymphoma classification accordingly the validated WHO scheme for veterinary medicine,<sup>15</sup> and an homogeneous treatment approach with an 19 week CHOP protocol, are characteristics that differentiated the present work from the previous heterogeneous studies.

The present work contributed for the rationale of blood cell ratios usage in veterinary medicine for prognostication despite the limitations of the automatic cell analysers, in cell identification and allocation when nucleated red blood cells are present.

The work here presented adds innovative and important information to better understand the epidemiology of canine lymphoma and particularly the clinical and prognostic spectrum of DLBCL in the dog. It contributes to the early studies in cDLBCL prognostic studies. The hereby-described prognostic information can be widely available, cost effective and quick to obtain, allowing risk stratification of patients and potentially tailored therapeutic approaches. A window to the putative contribution of *Leishmania spp.* to lymphomagenesis remains open and constitutes an avid research subject to pursue

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