Praca oryginalna *Original paper*

Predictive value of topoisomerase IIα immunoreactivity in canine diffuse large B-cell lymphoma (DLBCL) and peripheral T-cell lymphoma (PTCL) treated with a doxorubicin-based chemotherapy protocol

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Summary

Topoisomerase IIα (TOPIIα) plays a key role in DNA replication and is a molecular target for TOPIIα inhibitor chemotherapy. A doxorubicin-based chemotherapy remains the most effective treatment for canine lymphoma, but there are no clear indications for its use in individual patients. The aim of this study was to assess the predictive value of TOPIIα expression in the treatment of canine lymphomas with TOPIIα inhibitors. Samples of formalin-fixed paraffin-embedded lymph nodes from 14 dogs with diffuse large B-cell lymphoma (DLBCL) and 5 dogs with peripheral T-cell lymphoma (PTCL) treated with a CHOP protocol were immunohistochemically labelled with anti-TOPIIα. Progression-free survival time (PFS) and overall survival time (OS) were estimated and compared with TOPIIα expression. Dogs with DLBCL had a higher level of TOPIIα expression compared to dogs with PTCL. The median PFS for dogs with DLBCL was significantly longer (260 days) than it was for dogs with PTCL (60 days). In DLBCL dogs with a high TOPIIα expression level, the median PFS was significantly longer (310 days) then it was in dogs with week or medium TOPIIα expression (51 days). The results of the study indicate that the immunohistochemical assessment of TOPIIα expression may be a valuable predictive indicator for anthracycline-based treatment of dogs with malignant lymphoma.

Keywords: canine lymphoma, immunohistochemistry, topoisomerase IIα, CHOP protocol, chemotherapy

Canine lymphoma remains one of the most chemotherapy-responsive tumours in the dog (1, 3, 4, 6, 9, 16, 21, 33, 37, 38). The aim of the therapy is to achieve a complete and long-term remission in the shortest possible time, i.e. the disappearance of the clinical signs of the disease (manifested mainly by the reduction of lymph nodes to their physiological size). The effectiveness of treatment is influenced by many factors, the most important of which include the phenotype of the lymphoma, the clinical stage of the disease at the time of its diagnosis and the chemotherapy protocol used (5, 12, 14, 25, 29, 30, 34). The most common lymphomas in dogs are diffuse large B-cell lymphoma (DLBCL) and peripheral T-cell lymphoma (PTCL) (4, 6, 7, 8, 37). In the chemotherapy of malignant lymphoma in dogs, mostly multi-drug regimens are used, based on a long-term (at least 6 months) alternating use of several cytostatic drugs (2, 11, 22, 31, 36). High doses of cytostatics (induction therapy) are used in the first phase of treatment, and maintenance treatment is applied after remission has been achieved. The use of multi-drug chemotherapy makes it possible to achieve 6-10 months of remission in about 85% of patients, with an average survival time of 8 to 12 months. In some cases, therapy can extend a dog's lifespan up to 36 months (4, 9, 10, 26, 35). Despite the certain diagnosis, it is difficult to determine the prognosis, choose the optimal treatment method and predict the response to treatment in each individual patient. In addition, there is a possibility of side effects, such as bone marrow suppression, toxic liver damage and damage to the heart muscle, which

Tab. 1. 19-week CHOP chemotherapy protocol

Drugs and dosage	Weeks																		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Vincristine (0.7 mg/m² i.v.)	x		x			x		x			x				x				x
Cyclophosphamide (250 mg/m² p.o.)		x					x						x						
Prednisolone (mg/kg p.o.)	2	1	1	0.5															
Doxorubicin (30 mg/m² i.v.)				x					x								x		

complicate the treatment process (20). In order to determine the most effective therapy regimen, predictive factors are searched for, i.e. factors that would allow the appropriate selection of a chemotherapy regimen not only for a specific type of lymphoma, but for a specific patient (personalized therapy). Doxorubicin (Dox), belonging to the anthracycline group, exhibits a broad spectrum of antineoplastic activity and is used in the treatment of many types of neoplasia, including as a monotherapy drug or as one of the components of multiple CHOP therapy – cyclophosphamide, doxorubicin hydrochloride (hydroxydaunorubicin), vincristine sulfate (Oncovin sulfate), and prednisone in the treatment of canine non-Hodgkin's lymphoma (14). The mechanism of the antineoplastic activity of doxorubicin is based on the inhibition of the function of topoisomerase IIα (TOPIIα), an enzyme involved in cell division including transcription, recombination, replication, and chromosome segregation. The highest expression of TOPIIα is observed in the G2/M phase of the cell cycle, and the lowest at the end of mitosis (13, 23). Inhibition of topoisomerase IIα function by doxorubicin leads to the formation of numerous intra-strand bonds that block the basic processes of transcription and replication. Cells with damaged DNA are then eliminated by apoptosis (32).

The aim of this study was to assess the predictive value of TOPIIα expression in the treatment of canine lymphomas (DLBCL and PTCL) with the use of a doxorubicin-based chemotherapy protocol (CHOP).

Material and methods

Patient selection. The study involved a retrospective analysis of clinical outcomes of selected dogs diagnosed with DLBCL and PTCL at the Department of Pathomorphology and Forensic Veterinary Medicine at the University of Life Sciences in Lublin between February 2016 and October 2018. The histological type of the lymphoma was the criterion for inclusion in the research group. To be eligible for study entry, dogs had to satisfy the following criteria: having undergone a complete clinical staging consisting of patient history, physical examination, complete blood count, serum biochemistry profile, urinalysis, three-view thoracic radiographs and abdominal ultrasound, having been recently diagnosed with DLBCL or PTCL based on cytological features, histomorphological features and immunochistochemistry, having received at least one dose of doxorubicin included in a CHOP protocol as their first chemotherapy treatment, having been followed-up to the end of their life or for at least 12 months after initial treatment. Dogs were excluded from the study if they had received treatment with steroids prior to the administration of CHOP and had another serious medical illness.

Treatment. The dogs selected for the study were treated according to a CHOP protocol including the following drugs: vincristine, cyclophosphamide, prednisone and DOX. The schedule of the cytostatic drugs, their doses and the method of administration are presented in Table 1.

Histologic examination. Enlarged popliteal lymph nodes for histological and immunohistochemical evaluation were surgically resected from dogs prior to chemotherapy and immediately fixed in 10% neutral buffered formalin over 24 h, embedded in paraffin blocks and cut into sections, 4 μm thick. The haematoxylin and eosin stained tissue sections were evaluated according to WHO classification criteria (15, 28).

Immunohistochemistry. The immunohistochemical examination using mouse monoclonal primary antibody against human TOPIIα clone Ki-S1 (Dako Glostrup, Denmark) diluted 1 : 200 was carried out in order to asses TOPIIα expression. The lymphoma phenotype was determined using polyclonal rabbit anti-human CD3 antibody (Dako, Glostrup, Denmark) diluted 1 : 300 and monoclonal mouse anti-human CD79αcy antibody, clone HM57 (Dako, Glostrup, Denmark) diluted 1 : 100. Target antigen retrieval for CD3 was performed by immersion in proteinase K for 10 minutes, whereas heat-induced epitope retrieval for CD79α, and TOPIIα was performed with an automated pressure cooker in a citrate buffer, pH = 6.0. For immunohistochemical staining, a system of detection of antigen–antibody complexes was used based on secondary antibodies combined with biotin directed against mouse monoclonal primary antibodies LSAB plus horseradish peroxidase (HRP, K0690; Dako). The enzyme labelling the reaction site was HRP conjugated with streptavidin; tetrahydrochloride-3,-3-diaminobenzidine (DAB) was used as a chromogen (SK-4100; Vector Laboratories, Peterborough, UK). The sections were counterstained with Mayer's haematoxylin. In all immunohistochemical reactions, a double-control system was used: a negative control, in which incubation with the primary antibody was replaced with appropriate IgG sera, and a positive control, in which the incubation was carried out on palatine tonsil tissue with a proven positive reaction with the antibody. Morphometric measurements of immunohistochemically stained lymph node sections were performed using a computer image analysis system (NIS-Elements BR-2.20; Laboratory Imaging, Praha, Czech Republic). The evaluation of TOPIIα expression was performed by two independent pathologists, who analysed at least 500 cells in different fields of view at a magnification

of 400 ×. According to the scoring system proposed by Remmele et al. for estrogen receptors, the number of cells with a positive reaction was estimated, where 0 = no positive cells, 1 = ≤ 25% positive cells, 2 = 26-50% positive cells, 3 = 51-75% positive cells, 4 = more than 75% positive cells, and reaction intensity was evaluated, where 0 – no reaction, 1 – mild reaction, 2 – moderate reaction, 3 – intense reaction (27). The final result was a product of the parameters (percentage of positive cells × reaction colour intensity). Based on the number of points obtained and criteria proposed by Hajduk et al., the expression of TOPIIα was determined, where 0-1 meant no expression (–), 2-3 weak expression (+), 4-8 moderate expression (++), and 9-12 strong/high expression (+++) (13). For purposes of statistical analysis, animals with diagnosed lymphoma were classified into groups according to the level of TOPIIα expression. The lymphoma phenotype was determined by the percentage of the expression of CD3 and CD79α molecules (membrane and cytoplasmic reaction) in neoplastic cells in 10 fields of view under 40× magnification. B-cell lymphomas were diagnosed when more than 80% of cells expressed CD79α antigen, whereas T-cell lymphoma was diagnosed in cases where more than 80% of cells expressed CD3.

Follow-up study. All dogs were evaluated 7 days after the first administration of the COHP protocol to assess the response to treatment and then were followed up every 3 months for at least 1 year after the initial treatment to monitor for relapse and to determine progression-free survival (PFS) and overall survival (OS). In addition, the dogs were examined individually when the owners reported any problems. The follow-ups included a history, complete physical examinations, thoracic radiographs and abdominal ultrasound examination. When an animal died or was euthanized, a post-mortem examination was performed. Response to therapy was determined using the Veterinary Cooperative Oncology Group (VCOG) response evaluation criteria for lymphoma (34). A complete remission (CR) was defined as the disappearance of all measurable peripheral lymph nodes. A partial remission (PR) was defined as at least a 30% reduction in the sum of the longest diameters of peripheral lymph nodes measured at first treatment. Progressive disease (PD) was defined as a more than 30% increase in the sum of the longest diameters of measurable peripheral lymph nodes or the appearance of new lesions. Response duration was reported as progression-free survival time (PFS) and overall-survival time (OS). PFS was defined as the time from the initiation of the 15-week CHOP protocol to disease progression or death from any cause. OS was defined as the time from CHOP initiation to death from any cause.

Statistical analysis. The results obtained were analyzed statistically. The quantitative variables analyzed were presented as the mean value, median and standard deviation, whereas the qualitative variables were shown as the number and percentage. For the qualitative variables, the Chi^2 test (with Yates correction) was used to detect the existence of a relationship between them. The normality of the distribution of the variables in the groups was checked using the Shapiro-Wilk normality test. The Mann-Whitney U test was used to examine the differences in TOPIIα expression between the two groups. The study also used the Kaplan-Meier survival analysis, and the survival time in the groups was compared using the log-rank (Mantel-Cox) test. A significance level of $P < 0.05$ was adopted, indicating the existence of statistically significant differences or relationships. The Statistica 9.1 software (StatSoft, Poland) was used for the database and statistical research.

Results and discussion

Nineteen dogs treated with the CHOP protocol for non-Hodgkin's lymphoma were included in the study. The age of the dogs included in the study ranged from 3 to 14 years (mean = 6.9 years, SD = 2.9). The group included 8 males (42.1%) and 11 females (57.9%). The most common breeds in the study were German shepherds – 5 (26.3%), Boxers – 3 (15.8%) and mixed breed dogs – 3 (15.8%). The other breeds were represented by one individual each. Twelve dogs were in clinical stage III, and 7 dogs were in clinical stage IV. The cytopathological, histopathological and immunohistochemical examination revealed 14 B-cell lymphomas with histomorphological features characteristic of DLBCL and 5 T-cell lymphomas with morphology typical of PTCL. Information regarding signalement, age, sex, breed and stage of disease is presented in Table 2. The overall response rate (ORR) for 19 dogs treated with the CHOP protocol was 100%, with 8 (42.1%) dogs showing a complete remission (CR) and 11 (57.9%) dogs showing a partial remission (PR) as their best response. A total of 8 (57.14%) out of 14 dogs with DLBCL had CR, and 6 (42.86%) had PR, whereas all dogs with PTCL had PR. There was no significant difference in remission between the dogs with B-cell

Tab. 2. Baseline characteristics of the patient population (n = 19)

Parameters		n (%)
Age	Average – 6.9 (median 6.0) SD = 2.9	
Sex	Male	8 (42.1)
	Neutered male	3 (37.5)
	Female	11 (57.9)
	Spayed female	8 (72.73)
Weight	Mean (SD)	24 kg
Breed	German shepherd	5 (26.3)
	Boxer	3 (15.7)
	Mixed-breed	3 (15.7)
	American Staffordshire terrier	1 (5.3)
	Bernese mountain dog	1 (5.3)
	Daschshund	1 (5.3)
	French buldog	1 (5.3)
	German shorthaired pointer	1 (5.3)
	Golden retriever	1 (5.3)
	Schnauzer	1 (5.3)
	Shih tzu	1 (5.3)
Histologic classification/ immunofenotype	DLBCL	14 (73.7)
	PTCL	5 (26.3)
Clinical stage	III	12 (63.1)
	IV	7 (36.9)

and T-cell lymphoma (P = 0.0903) (Tab. 3). The median PFS for all dogs was 110 days (range: 34-920 days), whereas the median OS was 165 days (range: 70-1020). The median PFS was 260 days (range: 45-920) for dogs suffering from DLBCL and 60 days (range, 34-125) for dogs with PTCL. The median OS was 296 days (range: 70-1020) for dogs with DLBCL and 89 days (range, 80-183) for those with PTCL. Kaplan-Meyer analysis revealed statistical difference in PFS between dogs suffering from DLBCL and PTCL (P = 0.018), whereas the difference in OS was not significant (P = 0.090) (Tab. 4, Fig. 1).

TOPIIα expression was evident in all cases with a diffuse granular nuclear pattern of immunohistochemical reaction, although the number of positive cells and reaction intensity varied (17, 18). A high number of strongly positive cells were observed at the periphery of lymph nodes, whereas less numerous positive cells with weaker reaction were present in the central areas of lymph nodes. According to the established TOPIIα expression scoring system, cases with high TOPIIα expression (8 cases, 57.14%) and moderate expression (4 cases, 28.57%) were the most numerous in the DLBCL group, whereas in the PTCL group moderate expression predominated (4 cases, 80.00%). Nevertheless, the difference in TOPIIα expression level between DLBCL and PTCL groups of lymphomas was not significant (Tab. 3). Due to the small number of dogs in the group of dogs with PTCL, further analyses were performed only in the group of dogs with DLBCL, which was further subdivided into two groups: one group with weak and moderate TOPIIα expression and the other group with high TOP2α expression. In the group of dogs with DLBCL and high TOPIIα expression, 7 dogs (87.50%) had CR and only 1 dog (12.50%) had PR, while in the group with weak and moderate TOPIIα expression 5 dogs (83.33%) had PR and only one dog (16.67%) had CR. The relationship between the type of remission and the level of TOPIIα expression was significant (P = 0.0353) (Tab. 5). The median PFS for dogs suffering from DLBCL with high TOPIIα expression was

Tab. 3. Relationship between the morphological type of lymphoma and treatment response and TOPIIα expression

Group		DLBCL (n = 14) n (%)	PTCL (n = 5) n (%)	Statistical analysis
Response to therapy	PR CR	6 (42.86) 8 (57.14)	5 (100.00) 0 (0.00)	Chi^2_{Yates} = 2.869 P = 0.090
TOPIIα expression	+ ++ +++ Median range	2 (14.29) 4 (28.57) 8 (57.14) 11.29	1 (20.00) 4 (80.00) 0 (0.00) 6.40	U = 17 P = 0.107

Tab. 4. Survival time parameters by lymphoma type and TOPIIα expression*

	Group		M [± 95% CI]	Me [± 95% CI]	Log rank Chi^2, P
PFS	Total DBCL PTCL		222.9 [116-329.8] 278.3 [144.4-412.2] 67.8 [36.7-98.9]	110 [38.9-181.1] 260 [0.0-577.2] 60 [27.8-92.2]	Chi^2 = 5.635 P = 0.018
OS	Total DBCL PTCL		271.9 [157.5-386.3] 325.9 [180.6-471.2] 120.6 [77.4-163.8]	165 [51.2-278.8] 296 [0.0-596.7] 89 [82.6-95.4]	Chi^2 = 2.867 P = 0.090
PFS	DLBCL	Total TOPIIα expression +/++ +++	278.3 [144.4-412.2] 394.4 [31.6-215.4] 278.3 [203.3-585.4]	260 [0.0-577.2] 51 [43.8-58.2] 310 [268.4-351.6]	Chi^2 = 9.179 P = 0.002
OS	DLBCL	Total TOPIIα expression +/++ +++	325.9 [180.6-471.2] 456.6 [54.5-248.8] 325.9 [252.4-660.8]	296 [0.0-596.7] 76 [66.4-85.6] 370 [286.8-453.2]	Chi^2 = 9.140 P = 0.003

Explanation: * – DLBCL

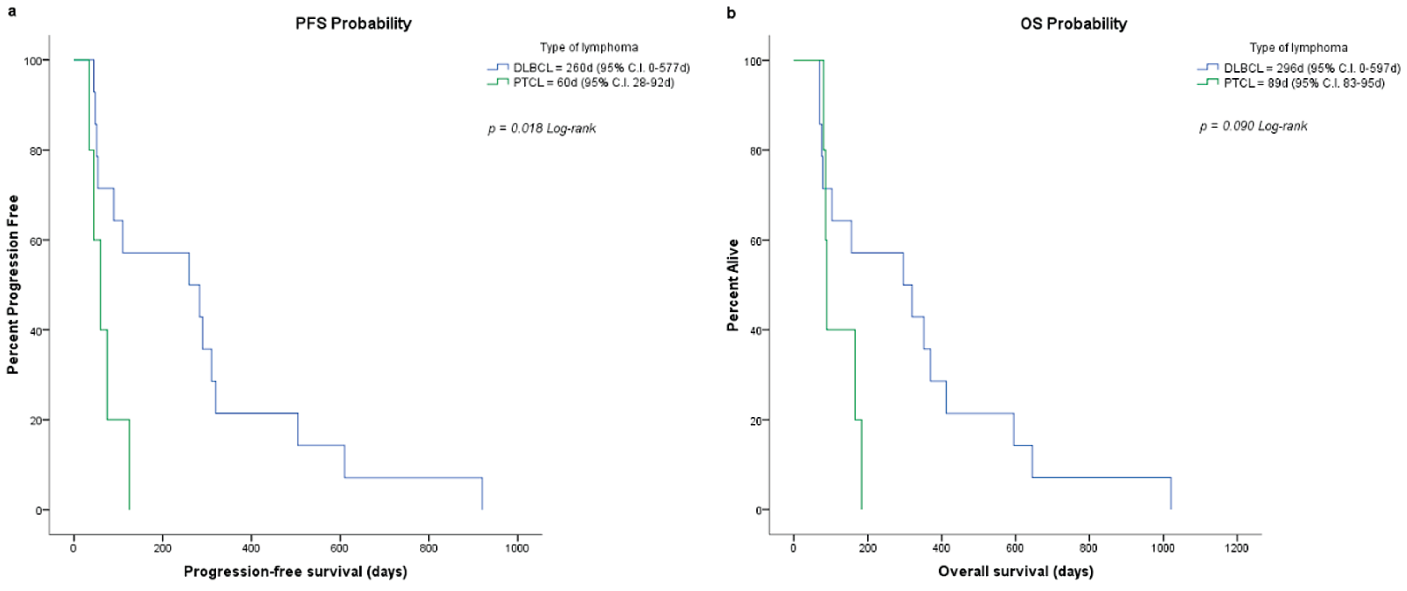

Fig. 1. Kaplan-Meier curves of PFS (a) and OS (b) for 19 dogs with canine lymphoma (DLBCL + PTCL) treated with CHOP

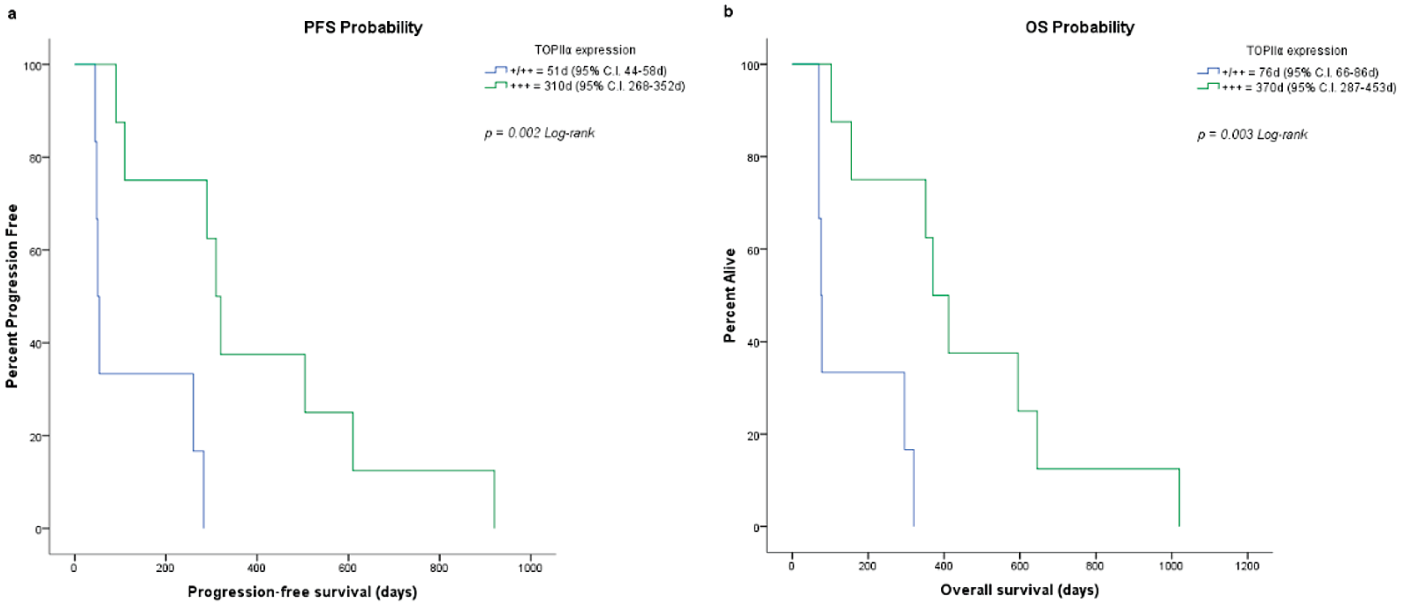

Fig. 2. Kaplan-Meier curves of PFS (a) and OS (b) for 14 dogs with DLBCL treated with CHOP

Tab. 5. Response to treatment depending on TOPIIα expression in the DLBCL group

Group	TOPIIα expression		Statistical analysis
	+/++ (n = 6) n (%)	+++ (n = 8) n (%)	
PR	5 (83.33)	1 (12.50)	$Chi^2_{Yates} = 4.430$ P = 0.035
CR	1 (16.67)	7 (87.50)	

310 days (range: 90-920) and the median OS was 370 days (range: 103-1020), whereas for dogs with weak and moderate TOPIIα expression the median PFS was 51 days (range: 34-310) and OS was 76 days (range: 70-370). Kaplan-Meyer analysis revealed significant differences in PFS (P = 0.002) and OS (P = 0.003) between dogs suffering from DLBCL with different TOPIIα expression levels (Tab. 4, Fig. 2).

According to epidemiological studies, lymphomas constitute up to 24% of all neoplasia in dogs, and the largest percentage are diffuse large B-cell lymphoma (DLBCL) and peripheral T-cell lymphoma (PTCL) (4, 6-8). These lymphomas are characterized by a high degree of malignancy and rapid progression. It is assumed that, in the absence of treatment, the average survival time of animals with lymphoma is 4 to 6 weeks (33). Appropriate therapy can extend the lifespan of an animal, significantly reducing or completely eliminating clinical signs. Lymphoproliferative diseases are classified as systemic diseases, and therefore the only effective method of therapy is pharmacological therapy with cytostatic drugs. The most effective are multi-drug regimens based on the alternating use of several cytostatics, among which the most frequently used regimen is CHOP (3-5, 9, 10). Many authors emphasize that among the drugs included in the CHOP protocol, doxorubicin is the most important one for the effectiveness of therapy (2, 12, 14). The research group consisted of 19 dogs, 11 females and 8 males. The dominant were middle-aged dogs (mean = 6.9 years), mainly large breeds (average weight = 24 kg). It is worth emphasizing that most dogs showed significant advancement at diagnosis (clinical stage III or IV according to the WHO classification, including lymphadenopathy and involvement of the spleen and/or liver). The above data are consistent with the epidemiological observations of other authors and indicate that the majority of lymphomas in dogs are diagnosed in the advanced stage (1, 2, 30). DLBCL was found in 14 animals and PTCL in 5 dogs. All dogs in the study group were treated according to the CHOP regimen and showed a 100% overall response rate (ORR). Complete remission (CR) of clinical signs was observed in 8 animals (42.1%) and partial remission (PR) in the remaining dogs. Dogs with DLBCL showed a significantly better response to treatment, with CR of 57.14% of all animals. In the group of dogs with PTCL, none of the animals achieved complete remission. Results in other studies were partially similar to those in our study. ORR in dogs with lymphoma treated with CHOP ranges between 84% and 100% (3, 4, 9, 10, 26). Other literature data, on the other hand, indicate a higher percentage of CR for both DLBC and PTCL. Slightly different CR values for DLBCL and PTCL were observed in the present study compared to results obtained by other authors. Complete remission ranges from 81.6% to 93% in dogs with DLBCL and from 12% to 88% in dogs with PTCL (1, 2, 26). In our study, the mean PFS for dogs from both study groups was 110 days (range: 34-920 days). In similar studies on T- and B-cell lymphomas, the mean PFS ranged from 140 to 245 days (range: 91-1795 days) (4, 9, 10, 12). In our study, significant differences in PFS and OS were found between the group of dogs with DLBCL and those with PTCL. Both PFS and OS for dogs with DLBCL were significantly longer than those for dogs

with PTCL treated with the CHOP regimen. These results are consistent with reports from many authors that the prognosis for survival and treatment effectiveness is much worse in T-cell lymphomas than it is in B-cell lymphomas (1, 2, 4, 9, 37). The next stage of the research was to determine the expression of TOPIIα in lymph node sections from both research groups. The positive expression of TOPIIα in tissue sections was observed in all cases, but the number of positive cells and the intensity of reaction varied. Among DLBCL, the majority (57.14%) were lymphomas with high TOPIIα expression, whereas in the PTCL group 80% were lymphomas with moderate TOPIIα expression. Due to the small size of the group of dogs with T-cell lymphoma, only dogs with DLBCL were qualified for further studies and divided into groups with high, low and medium expression of topoisomerase IIα. In the group of dogs with high TOPIIα expression, CR was observed in most animals, whereas in dogs with low or medium TOPIIα expression, PR predominated. A similar relationship was noted for PFS and OS. In dogs with high TOPIIα expression, both PFS and OS were significantly longer than in dogs with low or moderate TOPIIα expression. It seems reasonable that the variation in TOPIIα expression is related to clinical differences observed in response to treatment. Considering that TOPIIα is the target of molecular anthracycline drugs, including doxorubicin, it should be assumed that the effectiveness of treatment will increase with the increase in TOPIIα expression. Despite the fairly widespread use of anthracycline drugs in the treatment of lymphomas in dogs, no studies have been conducted so far on the relationship between treatment results and the level of TOPIIα expression, which makes it impossible to compare the results obtained in our study. In similar studies carried out by Pentheroudakis et al. on people with DLBCL, a positive correlation was observed between an increase in TOPIIα expression and an increase in response to treatment with anthracyclines (24). Among patients treated with the CHOP regimen, CR was achieved in 66.7% of patients with low TOPIIα expression and in 75.7% of patients with high TOPIIα expression. However, there were no statistically significant differences in PFS between the group of patients with low TOPIIα expression (where PFS was 37 months) and those with high TOPIIα expression (where PFS was 31 months). In patients with low TOPIIα expression, OS was relatively long, lasting up to 46 months, whereas in patients with high TOPIIα expression OS lasted 35 months. Patients with DLBCL and high TOPIIα expression showed a better response to treatment with the CHOP protocol, characterized by a faster onset of remission, but it did not extend their overall survival time (19, 24). Moreover, *in vitro* studies carried out on human tumour cell lines proved that cells with higher TOPIIα expression showed greater sensitivity to anthracyclines than those with low expression of this protein (24). In human medicine, studies on the prognostic and predictive value of TOPIIα expression were also conducted for breast cancer. This is due to the frequent use of doxorubicin as an adjunct to surgical treatment. As noted by Hajduk et al., the response to treatment with doxorubicin in patients with breast tumour and high TOPIIα expression was better than in patients with low expression of this protein. It was also found that the survival times of patients with low TOPIIα expression treated with doxorubicin were significantly lower than those of patients with high TOPIIα expression (19). It can be concluded that the assessment of the expression of TOPIIα in canine lymphomas may be helpful in selecting the optimal chemotherapy, and may thus be one of predictive parameters in assessing the possible response to anthracycline-based chemotherapy. However, further studies on a larger group of dogs are needed to assess the relationship between topoisomerase IIα expression and response to treatment in correlation with other prognostic and predictive indicators. The wide use of doxorubicin in animal oncology indicates the need to extend research to other types of neoplasms.

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