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Oxidative stress and antioxidant parameters in dogs with lymphoma during chemotherapy

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ABSTRACT

Purpose: Oxidative stress is a contributor in the development of neoplasms. OS is involved in multidrug resistance, adverse events, and outcome.

Methods: Forty- two dogs with lymphoma have been included in the study, 30 of which were treated by Cyclophosphamide - Hydroxyldaunorubicin® (doxorubicin) - Oncovin® (vincristine) - Prednisolone (CHOP)-based protocol. Tumor samples were excised for histopatholgy, immunophenotyping, Ki67%, and biochemical analyses. Thiobarbituric acid reactive substances (TBARS), reduced and oxidized glutathione (GSH, GSSG), glutathione peroxidase (GSH-Px), Cu-Zn-superoxide dismutase (SOD), ferric reducing ability (FRAP), vitamin-C, -E, retinyl palmitate and trans-retinol in red blood cell hemolysates, lymph node homogenates, and blood plasma have been measured. The results were correlated with the outcome of the chemotherapy.

Results: Median overall survival time (OST), relapse-free period (RFP) were 862 and 280 days, respectively, with adverse effects in 18 cases.

Plasma GSH levels increased with age (dogs from 1 to 6 years vs 9 to 13 years, $p = 0.0385$). Lymph node FRAP levels were higher in samples with higher Ki67%, and plasma GSH/GSSG ratio was lower in dogs with increased OST and RFP. Plasma retinyl palmitate levels were lower in dogs with increased RFP. Overall survival time was increased with lower lymph node TBARS (cut off: 53 nmol/mg protein), GHS-Px (cut off: 16 U/mg protein), and higher plasma all-trans-retinol (cut off: 4 mg/ml). Relapse-free period was increased with lower lymph node TBARS (cut off: 53 nmol/mg protein), lower FRAP (cut off: 6 µmol/mg) and higher SOD (cut off: 70 U/mg protein). During chemotherapy a gradual increase in plasma vitamin E and C concentrations was detected, while GSH and GSSG showed a decrease before adverse events.

Conclusion: Although oxidative stress parameters varied, some parameters, i.e. TBARS, GSH-Px, SOD, FRAP measured in lymph node samples, and plasma all-trans retinol might be used as prognostic indices.

Introduction:

Oxidative stress is developed by an imbalance between oxidizing and antioxidant agents. This mechanism is physiologically necessary for various processes such as aging, inflammation, synthesis of various biomolecules, such as thyroxin, prostaglandin derivates, steroid hormone generation, apoptosis, etc. However, these mechanisms work under proper antioxidant control by external and internal derivates, enzymes, and substrates. Some external stimuli can cause excessive oxidative stress which is harmful. These include effects of irradiation, oxidative agents (i.e. onions, paracetamol, nitrate pollution, etc.), hyperoxygenation, heavy metal pollution, etc. Some internal mechanisms can also induce severe symptoms, such as hypoxia reperfusion injury which is common in horses with large intestinal torsion or dogs with gastric dilation volvulus syndrome. Acute oxidative stress may result in degenerative processes and the emergence of pathological conditions.[1] Not only the increased formation of oxidative agents, such as reactive oxygen species (ROS) can result in damage but also decreased antioxidant defense. These problems are typical nutritional imbalances that commonly occur in production animals. These include vitamin E or selenium deficiency-induced myopathy in calves, and lambs, mulberry heart disease in swine, pulmonary hypertension syndrome, encephalopathy in poultry, and various liver dysfunctions in animals.

The targets of oxidative stress can be each basic biomolecules, such as nucleic acids, carbohydrates, proteins, and lipids.[2] Oxidative damage to proteins and DNA may have more long-term effects leading to mutations and carcinogenesis. ROS is considered an important member of carcinogens and has been implicated in the initiation, promotion, and progression of cancer. The association between chronic inflammation and cancer may be mediated via the release of ROS from leukocytes during i.e. inflammation.[3]

In general, high levels of oxidants can cause both DNA mutations and gene expression modifications that may give rise to the carcinogenic process.[4] It has been shown that the chemical activities of the different ROS can fragmentate single-ended double-strand DNA and hydroxylate DNA bases, thus causing genetic mutations and affecting transcription.[5]

Reactive Oxygen Species play an important role in microvascular blood flow modulation, vascular endothelial growth factor (VEGF) gene expression, and endothelial cell proliferation as well[6] Nitric oxide (NO) is the most important one of reactive nitrogen species (RNS). It is an intra- and intercellular messenger, active against bacterial infections, and promotes apoptosis, acts against cancer cell proliferation and metastasis. Moreover, at high concentration levels, NO is a potent cellular killer. Nevertheless, its positive effect on angiogenesis sometimes can be responsible for dichotomous roles[6].

In cancer patients, different oxidative mechanisms have been identified. 1) The mechanism is related to an altered energy metabolism and it is in connection with the general symptoms of the patient in an advanced state, such as anorexia, cachexia, nausea, and vomiting. This condition prevents a regular intake of substances such as glucose, proteins, and vitamins, resulting in an alteration of nutritional status and a significant accumulation of ROS. In advanced stages, an impaired insulin response can be associated with hyperglycemia and overproduction of free radicals.[7,8] 2) This process is related to chronic nonspecific activation of the immune system with excessive production of inflammatory cytokines which can, in turn, increase the production of ROS[7]. 3) The development of oxidative effects is related to the use of antineoplastic drugs. These can lead to an excessive production of free radicals[9]. Increased production of reactive species and the depletion of antioxidants can lead to oxidative stress. This is a relatively dangerous condition against cells and organic tissues.[10,11] On the other hand, oxidative stress mediates the effects of many cytostatic drugs by causing sublethal DNA damage and thus activating apoptosis [12]. To date, the role played by oxidative stress in veterinary oncology has been investigated, especially in lymphoma and mastocytoma[13,14,15], and resulted in different outcomes.

In our former study in canine patients, we found that lymphoma cases showed an impaired antioxidant status and free radical concentrations. These changes were correlated with higher proliferative character.

Besides, tumors with low oxidative burst capacity and higher reduced/oxidized glutathione ratio responded better to chemotherapy, and the affected blood and lymph nodes were under strong oxidative stress[13].

An increase in plasma isoprostanes and no changes in MDA (malondialdehyde) plasmatic concentration were reported in the study of Winter et al..[16] Both these molecules are intermediates and end products of lipid peroxidation induced by ROS. The d erivatives-reactive oxygen metabolites (d-ROMs) test does not detect the final products of oxidative stress but analyzes the pro-oxidant system measuring the production of ROS induced by certain conditions as the presence of iron. Therefore, d-ROMs test can detect alterations of the oxidant component earlier than isoprostanes and MDA[17].

However, authors found that there were no significant changes in oxidative status over time and, accordingly, no relationship with chemotherapy was observed. Also, clinical remission does not significantly affect the oxidative parameters.[18]

On the contrary, Winter's[16] results suggested that dogs with lymphoma have alterations in oxidant and antioxidant concentrations and that the status of some of these biomarkers normalizes after remission.

It is still not clear how much an increase in free radicals affects the growth and tumor progression and how much the development of the tumor can induce an increase in ROS.

Many studies have been carried out in humans and other species to obtain an overview of the oxidant and antioxidant activity in cancer patients but results are still very controversial.[7,12,19]

One of the most interesting previous observations concerns the significant correlation between the concentration of antioxidant enzymes, inflammatory proteins, and the clinical state of the patient. Moreover,

reports approve a prognostic role of oxidative stress parameters[7].

The prognostic role of oxidative stress in human lymphomas has also been studied, with conflicting results. According to some authors, the worst prognosis was those with decreased expression of antioxidant enzymes, but according to others, the conclusions were exactly the opposite. [2,21,21]

Even the use of chemotherapeutics can significantly affect the production of ROS and therefore, the consumption of antioxidants. Besides, a resistance to doxorubicin and vincristine has been linked to the increased amount of antioxidant intake, in particular, intracellular GSH.[22,23,24]

In a pilot study, authors proposed the use of oxidative markers during treatment with doxorubicin and emphasized the importance of a good reserve of antioxidants before the start of therapy.[25] A similar role has been suggested for the canine species by Vajdovich et al. (2005),[13] which showed that the response to chemotherapy was more effective in patients with a preserved oxidative balance.[13]

The oxidative changes may be affected by several factors as concomitant pathological conditions that may alter the oxidant-antioxidant balance.[26]

The inflammatory status is one of the main conditions of overproduction of ROS by neutrophils with a plasmatic increase of oxidative species.[11]

The controversy over the use of these biomarkers as prognostic indices led us to investigate this process from the first examinations until the end of chemotherapy protocols or the death of the animals.

We aimed to evaluate different oxidative stress and antioxidant parameters in dogs with lymphoma based on blood samples (plasma and red blood cell hemolysate) and lymph node samples naïve of therapy and then blood samples during CHOP-based chemotherapy.

Materials and Methods

SUBJECTS

Dogs with untreated multicentric lymphoma were presented to our oncology clinic at the University of Veterinary Medicine, Department of Clinical Pathology and Oncology for examination diagnosis and starting of chemotherapy. Altogether 41 dogs were included, and among them 31 were treated with chemotherapy.

Immunophenotype distribution was the following. B-cell types included diffuse Follicular Centre Cell (n=10), Diffuse Large B cell (n=19), MANTLE (n=1), B-cell Lymphoblastic Leukaemia/Lymphoma (n=2), B-lymphocytic (n=1), Lympho-plasmocytic (n=1) lymphomas. T-cell types were Peripheral T-cell (n=4), T-Lymphoblastic (n=1). Four dogs were in stage III/a of the disease, 3 in III/b, 14 in IV/a, 14 in IV/b, 2 in V/a and 2 in V/b.

Exclusion criteria included improper data collection (i.e. omitting control evaluations), discontinuation of treatment and the animal's death before starting chemotherapy, and chronic diseases unrelated to lymphoma. Staging was established by the WHO-system.[27]

Staging of the dogs was performed according to the WHO Lymphoma Classification System.[28] The dogs were in at least III or higher disease stage without other concomitant diseases (cardiac, renal, hepatic, neurologic disease). Moreover, the dogs should have been naïve to any preliminary therapy such as the administration of corticosteroids. Those dogs were excluded from the study which were fed with food highly supplemented in antioxidants. At that time dogs were not fed by those high-quality commercial diets in which antioxidant nutrients were specifically supplemented. All dogs underwent routine staging for lymphoma before initiation of chemotherapy, including baseline complete blood count, chemistry profile analysis, urinalysis, thoracic radiography, abdominal ultrasonography, bone marrow aspiration, and immunophenotyping.

The study was approved by the Institutional Animal Care and Use Committee of the University of Veterinary Medicine and all owners signed consent forms before enrolling their dogs in the study.

Treatment of patients was based on a modified Cyclophosphamide - Hydroxyldaunorubicin® (doxorubicin) - Oncovin® (vincristine) - Prednisolone (CHOP or COPA)-based protocol by Cotter and Goldstein (1987).[29] Dosages used in this protocol were: doxorubicin (Pharmacia and Upjohn S.p.A. Co., Milan, Italy) 30 mg/m$_2$: weeks 1, 9, 19; vincristine (Hospira UK Limited, Hurley, UK) 0.75 mg/m$_2$: weeks 2–9, then weeks 11–18; cyclophosphamide (Baxter Co., Deerfield, Illinois) 250 mg/m$_2$: weeks 4, 7, 13, 16; prednisolone (Actavis UK Limited, Devon, UK) PO SID: week 1 – 2 mg/bwkg, week 2 – 1.5 mg/bwkg, week 3 – 1 mg/bwkg and week 4 – 0.5 mg/bwkg. This protocol was used for a total of 19 weeks. If the patient showed relapse after the initial protocol (CHOP), further treatment options were discussed with the owner, and upon request the Madison-Wisconsin protocol[30] (L-CHOP) was initiated, starting with L-Asparaginase injection (Health Biotech Ltd. Chandigarh, India) once in a dose of 400 IU/bwkg SC. Eighteen dogs received CHOP-protocol, only as the first protocol, and 4 dogs received L-CHOP protocol, only as first protocol. Ten dogs received both (CHOP as first and L-CHOP as second). The treatment protocols were carried out by the oncology team of the faculty. The remission rate was estimated by measuring the size of the right prescapular lymph node using calipers at least in one largest dimension. Remission of lymph node size by 100% (complete remission) was recorded if the lymph node was normal in size or hardly palpable. The adverse treatment effects were graded according to the Veterinary Cooperative Oncology Group-Common Terminology Criteria for Adverse Events (VCOG-CTCAE v2).[31] If relapse was suspected, routine clinical examinations, fine needle aspiration (FNA) of lymph nodes, blood work, X-ray and abdominal ultrasonography were performed.

BIOCHEMISTRY AND HEMATOLOGY TESTS

Routine hematological and plasma biochemical parameters were determined in the blood samples weekly or every third week (in case of patients living in the countryside). These measurements were carried out in

the clinical laboratory of the Department and Clinic of Internal Medicine, University of Veterinary Medicine, Budapest, by using a hematology analyzer with veterinary software (Advia 120: Siemens Healthcare GmbH, Erlangen, Germany), and a clinical chemistry analyser (RX Daytona: Randox, Crumlin, UK) using commercial test kits (Diagnosticum Ltd., Budapest, Hungary). The reference values of different instruments were adjusted according to appropriate guidelines to obtain comparable results.[32] A qualitative blood count of each sample was performed using microscopic analysis of blood smears.

BLOOD SAMPLING AND PREPARATION

Blood samples (5 ml) were collected for antioxidants and biomarkers of oxidative stress assays at the time of staging and immediately before the initiation of chemotherapy. Blood samples were collected again each week when it was possible. If a treatment delay occurred blood sample collection for repeated oxidant and antioxidant levels was postponed such that collection occurred at the same point of the chemotherapy protocol. Baseline samples and those that were collected during chemotherapy were analyzed in the same assay.

Immediately after collection by introducing a peripheral venous access catheter into the *vena cephalica antebrachii*, a 2.5 ml, foil-covered ethylenediamine tetra acetic acid (EDTA) tube and a 5 ml foil-covered heparinized tube was filled with blood. EDTA samples were used for routine hematology analysis. Heparinized blood plasma samples were used for plasma oxidative and antioxidant measurements and for routine biochemistry. Tubes were processed within 15 minutes of collection. Heparinized tubes were centrifuged at 3000 RPM (1107 RCF) at 4 °C for 5 minutes. Plasma samples were snap-frozen and stored in separate aliquots at -80 °C until analysis. The remaining red cells in a heparinized tube were treated with 4ml of 0.9% NaCl, gently inverted several times, and centrifuged at 3,000 rpm at 4 °C for 5 minutes. After centrifugation, the saline and buffy coat portions were removed. This procedure was repeated twice, and 1 ml of the remaining washed red cells were diluted and hemolyzed in 2 ml of distilled water, and then frozen at -80 °C for further determination.

For plasma vitamin C analysis, a 50 ml-blood plasma aliquot was added to 150 ml of sample buffer (5 mM HPO_3, 5 mM Na-EDTA), centrifuged at 1000 g for 10 min at 4 °C. The supernatant was then stored at -80 °C until further analysis.

LYMPH NODE AND BONE MARROW SAMPLING AND PREPARATION

Lymph node and bone marrow samples were taken under general anesthesia. The dogs were anesthetized (propofol /AstraZeneca Co., Cambridge, UK / 5 mg/bwkg iv., isoflurane /Abbott Ltd., Budapest, Hungary / 1.5–2.5 V/V%, fentanyl / Gedeon Richter Plc., Budapest, Hungary / by constant rate infusion 0.01 to 0.04 mg/bwkg/h) and one of the enlarged (left prescapular) lymph nodes was excised for routine histological and immunohistochemical examination and further biochemical analysis.

Lymph node tissues were processed by their separation into two parts. One part was prepared for routine histological and immunohistochemical examination. At least 1 to 2 cm^3 of lymph node sections were cut from the excised tissue with care including the marginal and central areas. Incisions were made on tissues, at every 0.5 cm^3 wide area. These tissues were fixed by neutralized 4% formalin. Sections of (4%, neutralized) formalin-fixed paraffin-embedded lymph node tissues were kept at 56 C° for 12 hours in an incubator to prevent the detachment of sections from the silanized slides during antigen retrieval in the immunohistochemistry procedure.

Other parts of lymph node samples that were not formalin fixed were processed within 5 to 10 minutes after excision. Lymph node samples weighing 0.5 g were homogenized manually in 4.5 ml of Dulbecco 'A' phosphate buffered saline solution using Potter–Elvehjem tubes. Samples were centrifuged at 5000 RPM (2800 RCF) upper layers separated, then stored at -80 °C.

For lymph node vitamin C analysis, part of the lymph node samples was deproteinized by the addition of 3 ml ice-cold 5% trichloro acetic acid solution to 0.5-g lymph node tissue.

Bone marrow aspirates were taken for cytological analysis with a Jamshidi needle from the iliac crest (*crista iliaca externa*). The aspirates were smeared and stained with the conventional panoptic (Romanowsky type) staining kit (REAGENS Ltd., Budapest, Hungary).

HISTOPATHOLOGY AND IMMUNOHISTOCHEMISTRY

Tissues were routinely processed and sectioned at 3 µm and stained with hematoxylin and eosin. Serial sections of each case were placed on positively charged slides for further immunohistochemical labeling. All cases were labeled immunohistochemically for B and T cell antigens following a routine protocol. For immunophenotyping, CD3 was used for T-cell and CD79a for B-cell labeling by using rabbit anti-CD3 or mouse anti-CD79a antibody, respectively (DAKO Ltd., High Wycombe, UK). After diagnosing lymphoma, the tumors were graded according to the WHO classification scheme applied for canine lymphoma[27]. The cases were grouped into diagnostic categories: low-grade B-cell, high-grade B- and T-cell, low-grade T-cell, moreover, we subdivided the types as well.[33] Grouping was determined by histological grade (based on mitotic rate/400 field, with low-grade 0–5, intermediate 6–10, and high-grade >10).[33] The grading system was based on the original article written in 1986[34] with additional further suggestions.[33] To assess the proliferation status of the tumor, the percentage of Ki67-positive cells was calculated after immunostaining the sections with the Ki67 marker MIB-118 (DAKO Ltd., High Wycombe, UK). Approximately, a minimum of 150 (up to 500) cells were counted on each slide in 5 different zones of the section.

MEASUREMENT OF OXIDATIVE STRESS AND ANTIOXIDANT PARAMETERS

The following parameters were analyzed in blood plasma, red blood cell hemolysate and lymph nodes homogenates: Red blood cell hemolysate (RBC): RBC-SOD u/g prot., RBC-TBARS nmol/g prot. RBC-GH-Px U/g prot., RBC-GSH mmol/g prot., RBC-GSSG µmol/g prot., RBC-GSH/GSSG

Plasma (P): P-GSH µmol/l, P-GSSG µmol/l, P-GSH/GSSG, P-FRAP µmol/l, P-DL-alpha-tocopherol mg/ml, P-vit-C mg/ml, P-all-trans-retinol mg/ml, P-retinil-palmitate mg/ml

Lymph node (LN): LN-GSH-Px U/mg prot., LN-SOD U/mg prot., LN-FRAP µmol/mg prot., LN-TBARS nmol/mg prot. Chemicals were purchased from Sigma-Aldrich Co. (Budapest, Hungary) unless indicated otherwise.

Thiobarbituric acid reactive substances (TBARS) were measured in RBC hemolysates, plasma and lymph node samples.[35,36] Malondialdehyde (MDA) is one of the end products of the peroxidation of polyunsaturated fatty acids and one of the most important thiobarbituric reactive substances. The essence of its detection is that at high temperatures (90 to 100 °C) MDA can react with thiobarbituric acid (TBA). During the reaction, a rose-red, water-soluble product is produced, the amount of which can be measured with a spectrophotometer at a wavelength of 532 nm. MDA Standard was used for QC-testing (OxiSelect™ TBARS Assay Kit, MDA Quantitation, Part No. 233001, Cell Biolabs, San Diego, USA). Inter- and intra-assay coefficients of variation were 2.4 and 5.9%.

Reduced (GSH) and oxidized (GSSG) glutathione concentrations were measured in RBC hemolysates, plasma and lymph node samples based on the original methods described by Sedlak et al. (1968)[37] modified by Kosower et al. [38] and Matkovics at al.[35] in 1988. The method is based on the reaction of Ellmann's reagent (5,5'-dithiobis-2-nitrobenzoic acid, DTNB) with the sulfhydryl (SH-) groups in an alkaline medium and produces a yellowish product that can be measured with a spectrophotometer at a wavelength of 412 nm. SH groups can be contained in several molecules in red blood cells, but they mainly come from GSH. To determine GSSG, first it is necessary to determine GSH concentration, then by adding glutathione reductase and $NADPH+H^+$. By this reaction, all glutathione molecules will be reduced, and the total glutathione concentration is determined. The difference between the total glutathione and the previously measured GSH concentration gives twice the GSSG concentration. Oxidized (Sigma-Aldrich, Catalog Number G4501) and reduced L-glutathione (Sigma-Aldrich, Catalog Number: G4251) was used for QC. Inter- and intra-assay coefficients of variation for GSH and GSSG were 5.4 and 7.2%; 7.3, and 9.5%, respectively. In the tables, GSH concentrations are expressed by dividing the results by 1000, therefore the units are expressed in mmol/g prot.

The total antioxidant status was assessed based on the ferric-reducing ability of plasma (Ferric reducing anti-oxidant power, FRAP) described by Benzie et al. (1996).[39] FRAP was measured in blood plasma and lymph node samples. The principle of the method is the following. If a reducing agent is added to the iron (III)-tripyridyltriazine (Fe3+ -TPTZ) complex in a low pH medium, the final product, iron (II)- tripyridyltriazine (Fe2+ -TPTZ) solution will gain violet-blue color, which can be measured spectrophotometrically at the absorption maximum of 593 nm. The extinction value is directly proportional to the reducing power of the sample. FRAP positive control was used for QC (Sigma Aldrich, Catalog Number: MAK369E). Inter- and intra-assay coefficients of variation for GSH and GSSG was 11.3 and 12.5%.

Vitamin C (ascorbic acid) levels were measured using the modified method of Harapanhalli et al. (1993) or blood plasma samples.[40] Sample preparation: 100 µl plasma was mixed with 50 µl trichloroacetic acid (28 m%) in an Eppendorf tube. After addition of 50 µl dithiothreitol-metaphosphoric acid solution (5 mM metaphosphoric acid, 5 mM Na_2EDTA, 5 mM DL-dithiothreitol), the mixture was centrifuged at 9021 RPM (10008 RCF), 4°C for 10 min. The measurements were carried out by HPLC (Gilson pump and UV detector, Middleton, WI, USA) using methanol-water (95:5) as eluent. L-ascorbic acid analytical standard (Supelco, 47863) was used for QC. Inter- and intra-assay coefficients of variation were 3.3 and 7.1%.

Vitamin E (DL-alpha-tocopherol), all-trans-retinol, and retinyl palmitate concentrations in blood plasma were measured by modifying the method of Barbas et al. (1997).[41] Sample preparation: 100 µl plasma was mixed with 100 µl 0.1 M sodium dodecyl sulfate in an Eppendorf tube. After addition of 333 µl absolute ethanol and 333 µl hexane, the samples were centrifuged at 3000 g, 4°C for 5 min and the organic phases were collected. The leftover plasma was re-extracted with 333 µl hexane altogether 4 times, and the organic phases were pooled, evaporated under a nitrogen stream, and resolved. The measurements were done by HPLC using methanol-water (99:1) as eluent. Detection was carried out using a fluorometer (FS 970 Spectrofluoro Monitor, Kratos Analytical Instruments, Ramsey, NJ, USA) and quantified using DL-alpha-tocopherol (Merck, Darmstadt, Germany), all-trans-retinol and retinyl palmitate as reference compounds (Merck, Darmstadt, Germany). DL-alpha-Tocopherol acetate standard (47786, Supelco) was used for QC of vitamin E. Inter/intra-assay coefficients of variation were 6.2 and 4.1 %. Retinol (R7632, Sigma-Aldrich) was used for the QC of all-trans-retinol. Inter/intra-assay coefficients of variation were 8.2 and 10.1 %. Retinyl palmitate (Vitamin A palmitate) (PHR1235, Supelco) was used for the QC of retinil palmitate. Inter/intra-assay coefficients of variation were 5.8 and 9.9 %.

Glutathione peroxidase (EC: 1.11.1.9) (GSH-Px) activity was measured based on that GSH-Px promotes the oxidation of reduced glutathione (GSH) in the presence of cumene hydroperoxide.[42,43] Blood plasma, RBC-hemolysates, and lymph node samples were analyzed by using commercially available kits (Ransel kit; Randox, Cork, Ireland, Cat No: RS505). In the presence of glutathione reductase (GR), oxidized glutathione (GSSG) is converted back into its reduced form, for which hydrogen is provided by the coupled reaction NADPH+H+ ®NADP+. The reaction kinetics is monitored by measuring the decrease in extinction at 340 nm. Ransel (glutathione peroxidase) control solution was used for QC. Inter- and intra-assay coefficients of variation for GSHPx were 11.6 and 7.5%.

Superoxide dismutase (EC: 1.15.1.1) (SOD) activities were measured in blood plasma, RBC-hemolysates, and lymph node samples analyzed by using commercially

available kits (Ransod kit; Randox, Cork, Ireland, Cat No: SD126).[44] Xanthine in the reagent produces uric acid and superoxide anion radicals under the action of xanthine oxidase. The superoxide anion radical reacts with p-iodonitrotetrazolium (INT) forming red formazan. SOD in the sample competes with INT for the superoxide radical. The reaction kinetics is monitored by measuring the decrease in extinction at 505 nm. Ransod (superoxide dismutase) control was used for QC. Inter- and intra-assay coefficients of variation for SOD were 12.6 and 8.7%.

Results are given per gram protein content of the samples, which was determined spectrophotometrically using a Bio-Rad protein reagent (Bio-Rad Laboratories Ltd., California, USA) and following the manufacturers recommended protocol.

STATISTICAL ANALYSIS

Differences between groups were analyzed using the nonparametric linear regression analysis of Pearson's correlation, Kolmogorov–Smirnov test was used to check whether the populations have normal distribution, Mann–Whitney U test and Kolmogorov–Smirnov test was used as non-parametric tests, and ANOVA, and unpaired Student's t-test was used for data of normal (Gaussian distribution). Kaplan-Meier survival curves and log-rank tests were used for the survival analyses. The groups were compared in aspects of relapse-free period (RFP) and overall survival times (OST). P-values below 0.05 were considered significant. Statistical analysis was carried out using Microsoft Excel 2010, R version 3.0.20 (Foundation for Statistical Computing, Vienna, Austria), Stats Direct Statistical Software version 3.0.194 (StatsDirect Ltd, Altrincham, UK). Normal range calculation was performed by Reference guide for Reference Value Advisor. 1. Copyright (c) (Office 2010-2013, National Veterinary School of Toulouse).

Results

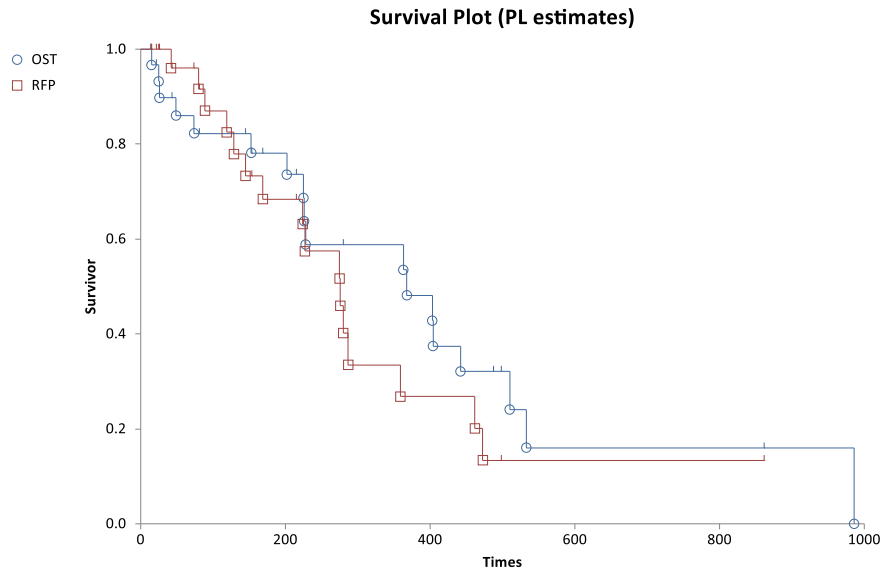
SUBJECTS AND CLINICAL FINDINGS

There were 21 male and 20 female dogs examined. Their age was mean: 6,84 (±2,29) years. Breeds of the treated and untreated male and female dogs were mongrel (n=8 and n=3, respectively), German shepherd (n=4 and n=2), Hungarian Vizsla (n=1 and n=1), Bullmastiff (n=1), Doberman pincher (n=3), Rottweiler (n=1 and n=1), Argentine mastiff (n=2), Bernese mountain dog (n=1), Russian black terrier (n=1), Staffordshire terrier (n=1 and n=2, respectively), Dachshund (n=1), Chow-chow (n=1), English pointer (n=1), Cocker spaniel (n=1 and n=1), Beagle (n=1), Hovawart (n=1), Sarplaniac (n=1), Bull terrier (n=1).

Out of the 41 dogs, 31 were treated by modified COPA and among them 5 received Madison-Wisconsin protocol. The 10 non-treated dogs were examined, but either the follow-up of treatment was problematic (n=3), or the dogs went into deteriorated state (n=2), or the owners refused treatment (n=5). The mean age of dogs was 6.84 (±2.32, median: 7.01) years.

Among the treated dogs median OST was 368 days (mean: 403.66, median lower 95% CI: 226, median upper 95% CI: 498 days, 13 censored). Relapse free period was 276 days (Mean: 329.4, median lower 95% CI: 169, median upper 95% CI: 287 days, 15 censored) (Log rank: P = 0.3979) (Figure 1). The 1-year survival was 32.25%, two years survival was 6.45 %. The half-year relapse was 45.16 %, and the 1-year relapse was 12.9 %.

Figure 1. Overall survival time (OST) and relapse free period (RFP) in 31 treated dogs with lymphoma



The age distribution was the following. Young: 1-6 years, middle aged: >6-9 years, old: >9-13 years (n=17; n=16; n=8, respectively; and 41,46%, 39,02%, 19,51%, respectively). Median OST of the dogs according to different age groups was the following: young: 403, middle aged: 986, old: 368 days (p= 0.768). Median RFP of the dogs according to different age groups was the following: young: 227, middle aged: 275, ld: 276 days (p= 0.779). Male dogs lived longer than females (403 vs 225 days, respectively, p=0.112), but relapsed earlier than females (227 days vs 276, respectively, p=0.977).

Overall survival time of the dogs according to different stage groups was the following: III: 25, IV: 510, V: 368 days (p= 0.308). Relapse free period of the dogs

according to different stage groups was the following: III: 227, IV: 473, V: 224 (p= 0.203).

Overall survival time of the dogs according to different substage groups was the following: a: 363, b: 368 days, respectively (p= 0.831), OST mean: 422,03 and 328,58, respectively. Relapse free period of the dogs according to different substage groups was the following: a: 275, b: 276 days, respectively. Relapse free period means were 302.44 and 306.46 days (p= 0.098).

Among the 31 treated dogs, adverse effects were documented during the 1st year of therapy, these included fever (n=5), neutropenia (n=6), anorexia (n=7), vomiting (n=4), diarrhea (n=6), dermatitis (n=2), cystitis (n=2). Altogether 18 dogs (58.1 %) had adverse effects. Grade distribution was grade 3 in 8, grade 2 in 4, and grade 1 in 6 dogs (25.81%, 12.90 %, 19.35%, respectively. In 6 out of 18 dogs with adverse effects showed these events within the 1st month of therapy.

Patients with T-cell lymphoma (n=5) lived shorter, vs patients with lymphoma of B-cell type (n=26) (median 202 and 404, p=0.365). The patients with T-cell lymphoma (n=5) had an earlier relapse, vs patients with lymphoma of B-cell type (n=26) (median 276 and 462, p=0.792). T-cell immunophenotype occurred significantly more in females (100%) than B-cell phenotype (41.66%) (p=0,014).

The Ki67% in the lymph nodes of all examined dogs was mean: 30.38 ±12.01, median: 44.00. Patients with Ki67% < 20% lived longer and relapsed later than those with Ki67%> 20% (OST: 403 and 363 days, respectively, p=0.935; RFP: 276 and 275, respectively, p=0.82).

The measured oxidative stress and antioxidant parameters did not differ significantly between groups of T and B immunophenotype; therefore, the reference ranges could be calculated, which are found at the Appendix.

OXIDATIVE STRESS PARAMETERS IN DIFFERENT LYMPHOMA BEARING DOG GROUPS

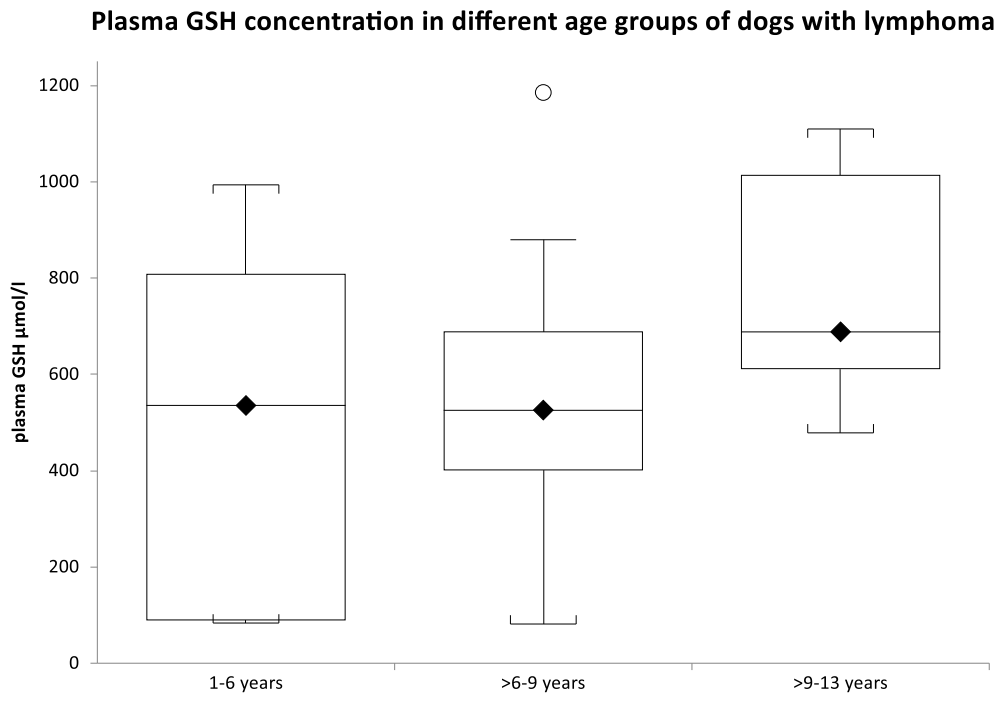
We calculated the reference ranges of our measured parameters, indicating the number on measured parameters in each cases, plasma (P), red blood cell hemolysate (RBC) and lymph node (LN) (Appendix).

In different age groups plasma GSH concentration (µmol/l) differed between the young (1 to 6 years), which had lower values, and old (9 to 13 years) dogs (P = 0.0385), while values of the middle aged (6 to 9 years) dogs (almost significantly) differed from the old ones (Table 1, Figure 1).

Table 1. Plasma GSH-concentration in different age groups of dogs

Age	Young (1 to 6 years)	Middle (6 to 9 years)	Old (9 to 13 years)
Plasma GSH mean (µmol/l)	498.10	534.19	762.6
Standard deviation (±)	336.9	304.8	223.6
P values	Young vs Middle	0.7743	
	Young vs Old	0.0385	
	Middle vs Old	0.0622	

Figure 1. Plasma GSH-concentration in different age groups of dogs

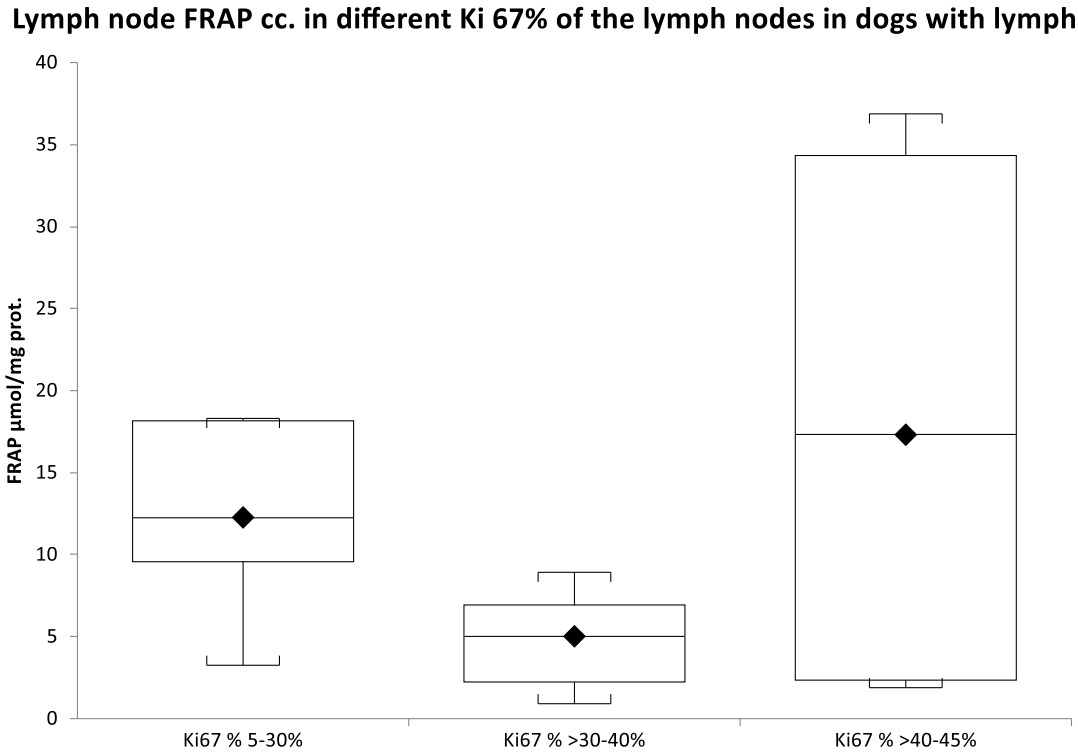


Ferric reducing anti-oxidant power values showed differences in lymph nodes (µmol/mg prot) of patients in groups with different Ki67% , as the lymph nodes with low proliferation rate (5 to 30%) showed slightly higher FRAP values than those with middle proliferation rate (> 30 to 40%). However, the lymph nodes with high proliferation rate (> 40 to 45 %) showed the highest values, with high standard deviation (Table 2, Figure 2).

Table 2. Lymph node FRAP values (µmol/mg prot) in groups with different Ki67 %

Ki 67 %	Low (5 to 30%)	Middle (> 30 to 40%)	High (> 40 to 45 %)
Lymph node FRAP mean (µmol/mg prot)	12.72	4.78	18.34
Standard deviation (±)	6.15	2.79	18.58
P values	Low vs Middle	0.041	
	Low vs High	0.597	
	Middle vs High	0.240	

Figure 2. Lymph node FRAP values (µmol/mg prot) in groups with different Ki67 %



EFFECTS OF OXIDATIVE STRESS PARAMETERS ON SURVIVAL DATA

Dogs have been distributed into different groups according to overall survival times and relapse free periods with significant differences found in plasma GSH/GSSG values. Dogs with shorter survival and relapse free period had higher GSH/GSSG values than dogs with middle and longer survival and relapse free values (Table 3, Figure 3 and Table 4, Figure 4).

Table 3. Changes of plasma GSH/GSSG values in dog groups distributed according to different overall survival times

OST	Short (7 to 180 days)	Middle (180 to 365 days)	Long (365 to 990 days)
Plasma GSH/GSSG mean	21.49	14.67	7.20
Standard deviation (±)	19.06	15.84	5.17
P values	Short vs Middle	0.5465	
	Short vs Long	0.0772	
	Middle vs Lon	0.161	

Figure 3. Changes of plasma GSH/GSSG values in dog groups distributed according to different overall survival times (OST)

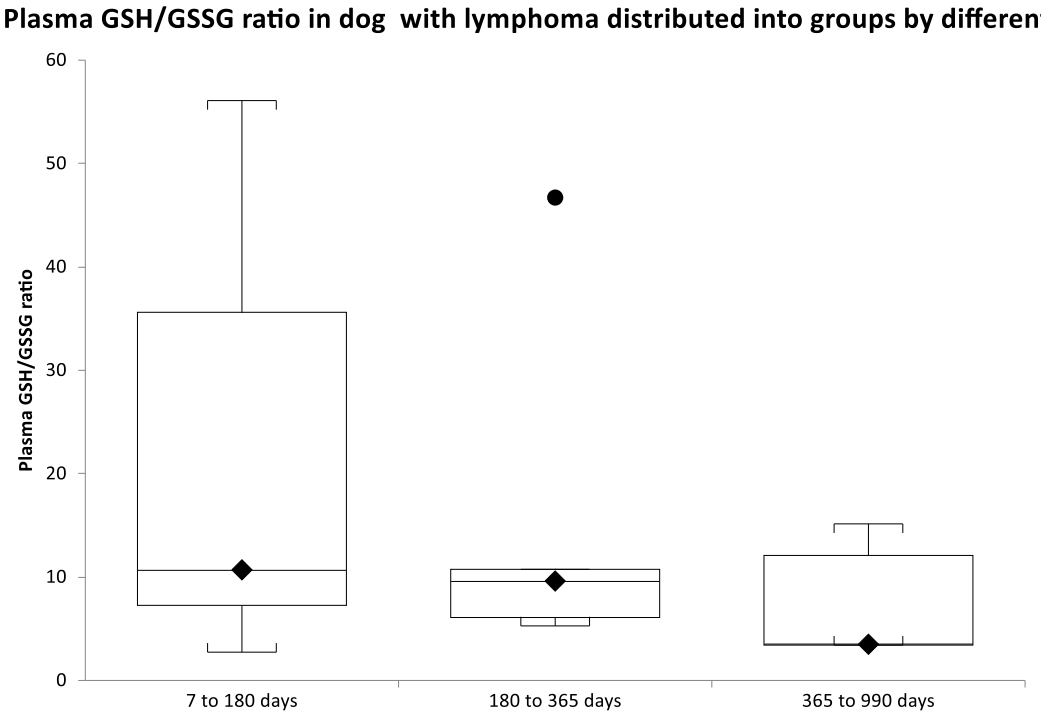
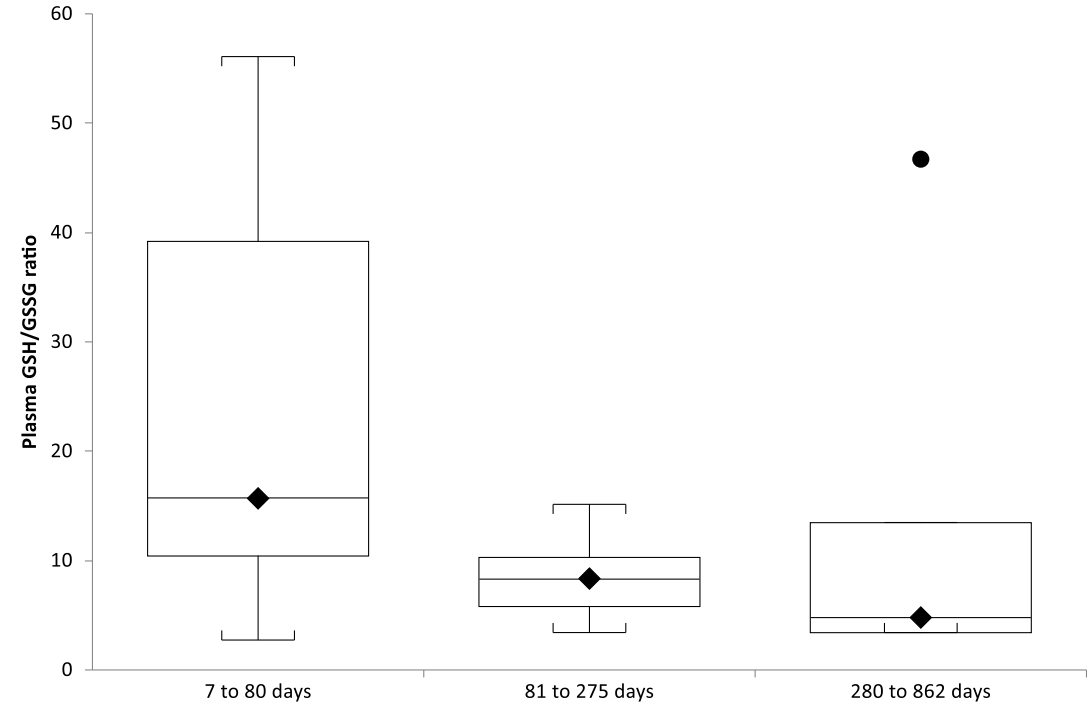


Table 4. Changes of plasma GSH/GSSG values in dog groups distributed according to different relapse free periods

RFP	Short (7 to 180 days)	Middle (180 to 365 days)	Long (365 to 990 days)
Plasma GSH/GSSG mean	8.02	5.29	7.14
Standard deviation (±)	2.09	1.85	2.76
P values	Short vs Middle	0.0024	
	Short vs Long	0.4404	
	Middle vs. Long	0.111	

Figure 4. Changes of plasma GSH/GSSG values in dog groups distributed according to different relapse free periods (RFPs)

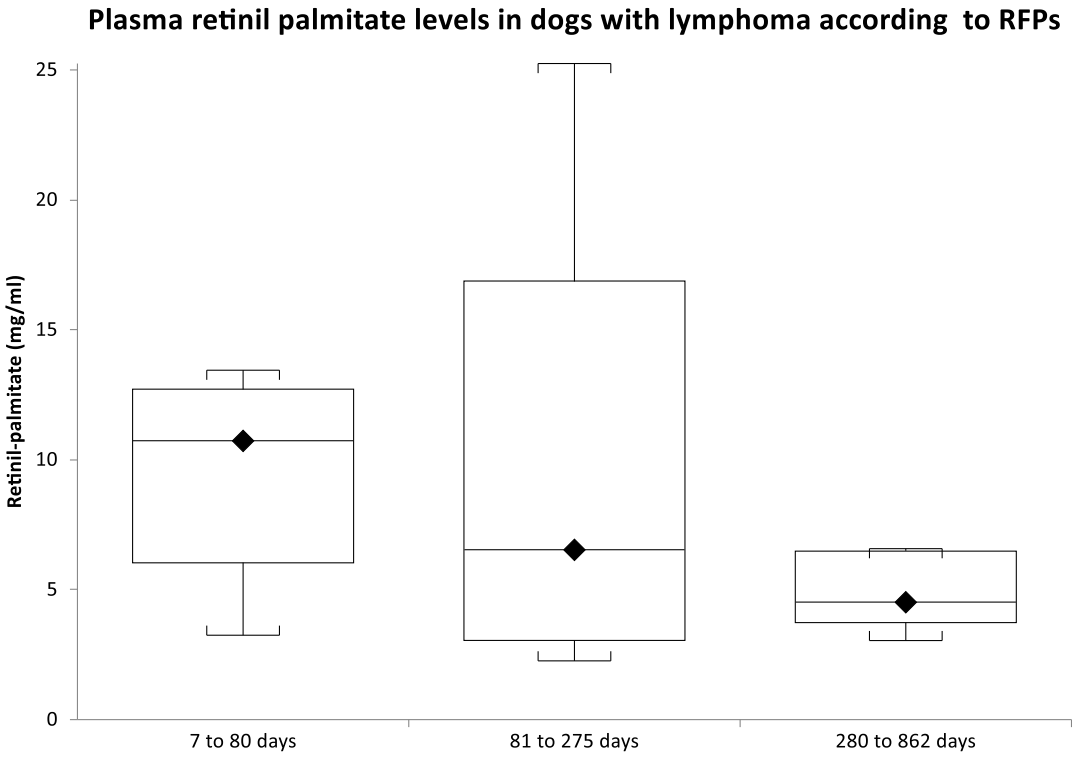


Plasma retinil palmitate (mg/ml) levels also differed in dogs distributed into different groups according to their relapse free periods. Higher values were detected in dogs with shorter relapse free period than in those with longer RFPs (Table 5, Figure5).

Table 5. Plasma retinil palmitate (mg/ml) levels in groups distributed according to different relapse free periods (RFPs).

RFP	Short (7 to 180 days)	Middle (180 to 365 days)	Long (365 to 990 days)
Plasma GSH/GSSG mean	8.02	5.29	7.14
Standard deviation (±)	2.09	1.85	2.76
P values	Short vs Middle	0.0024	
	Short vs Long	0.4404	
	Middle vs. Long	0.111	

Figure 5. Plasma retinil palmitate (mg/ml) levels in groups distributed according to different relapse free periods (RFPs).

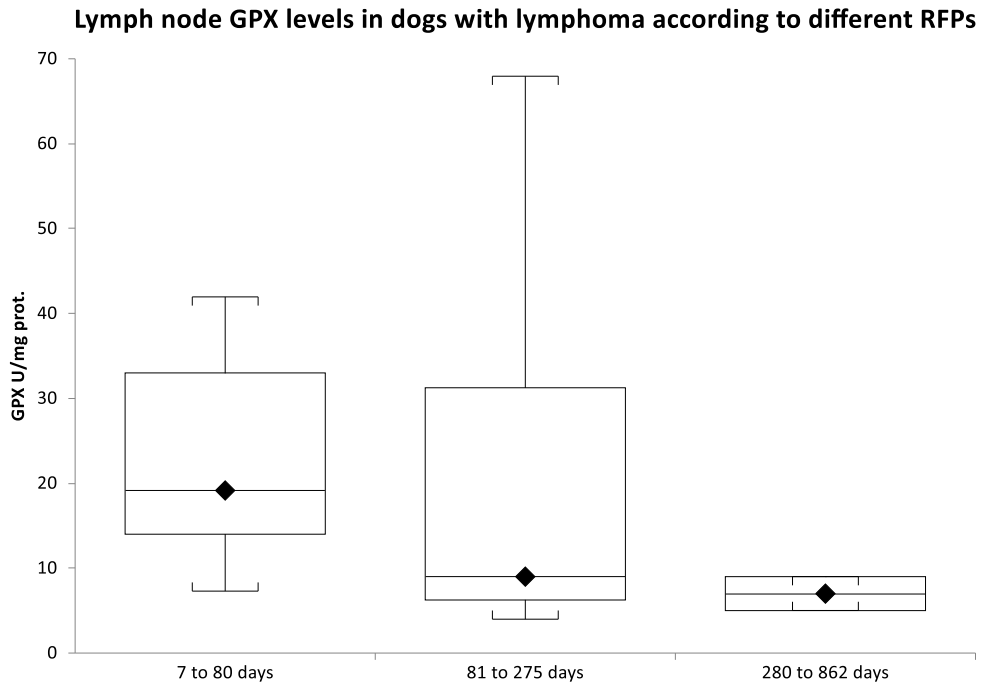


Lymph node GSH-Px activity (U/mg prot.) levels also differed in dogs distributed by different relapse-free periods. Higher values were detected in dogs with shorter relapse free period than in those with longer RFPs (Table 6, Figure 6).

Table 6. Lymph node GSH-Px activity (U/mg prot.) levels in groups distributed according to different relapse free periods (RFPs)

RFP	Short (7 to 180 days)	Middle (180 to 365 days)	Long (365 to 990 days)
Lymph node GHS-Px (U/mg prot.) mean	22.43	21.40	7.00
Standard deviation (±)	12.85	26.65	2.83
P values	Short vs Middle	0.9345	
	Short vs Long	0.0337	
	Middle vs. Long	0.5032	

Figure 6. Lymph node GSH-Px activity (U/mg prot.) levels in groups distributed according to different relapse free periods (RFPs)



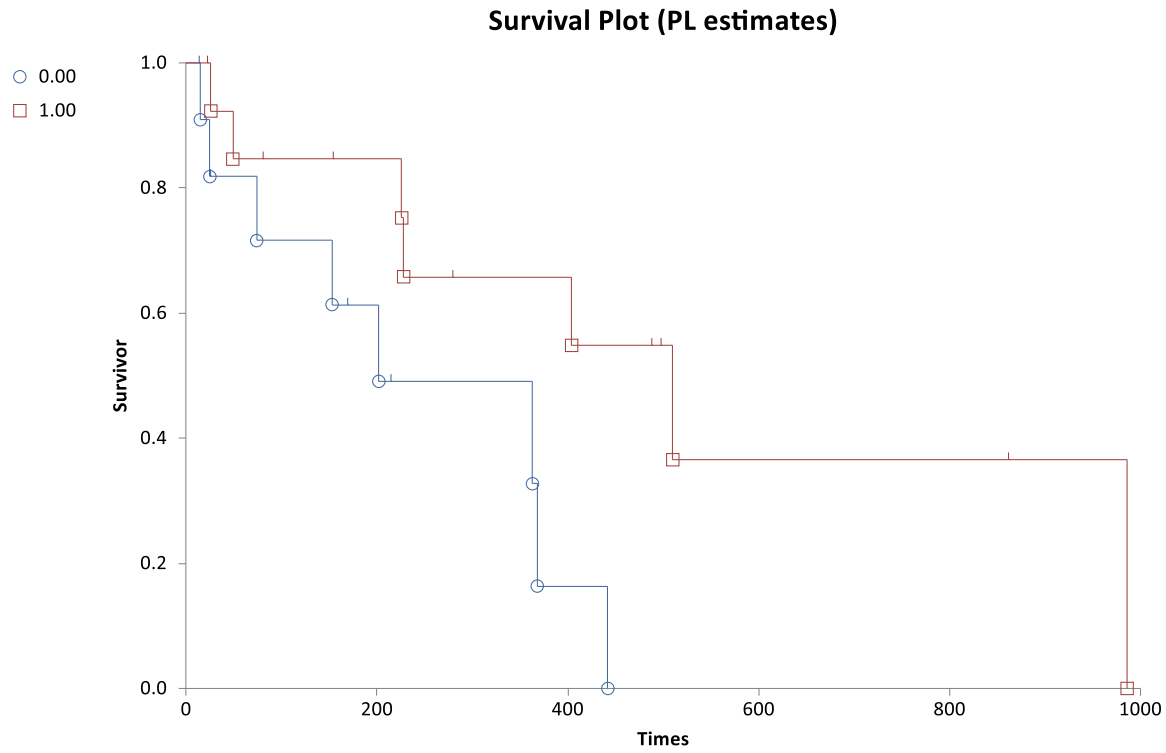
Lower vitamin A (all-trans retinol) concentration with a cut off 4 mg/ml was associated with shorter overall survival time (Table 7, Figure 7), while higher lymph node TBARS (cut off: 53 nmol/mg prot) and GSH-Px (cut off: < 16 U/mg prot) was associated with shorter OST (Table 8).

Table 7. Effects of plasma (P) vitamin A (all-trans retinol), lymph node (LN) TBARS and GSH-Px values on the overall survival time (OST) of dogs with lymphoma

	Mean estimate	Median estimate	Median lower 95% CI	Median upper 95% CI		
LN-TBARS < 53 nmol/mg prot.	756.13	can not estimate				
LN-TBARS > 53 nmol/mg prot.	174,4	153	26	202	Log Rank	0.033
P-All-trans-retinol < 4 mg/mL	243,58	202	74	363		
P-All-trans-retinol > 4 mg/mL	546,50	510	228	862	Log Rank	0.038
LN-GSH-Px < 16 U/mg prot.	597.17	510	510	862		
LN-GSH-Px > 16 U/mg prot.	146,8	49	15	202	Log Rank	0.003

Figure 7.

Kapplan-Meyer curve of the OST of dogs with different plasma all-trans retinol concentrations (0: < 4 mg/ml vs. 1: > 4 mg/ml) "0": P-All-trans-retinol < 4 mg/mL; "1": P-All-trans-retinol > 4 mg/mL



Lower lymph node TBARS concentration (cut off < 53 nmol/mg prot), lymph node FRAP (cut off: 6 µmol/mg) was associated with longer relapse free period, while lower lymph nodes SOD (cut off: 70 U/mg prot.) values weres associated with shorter RFP (Table 8).

Table 8. Effects of lymph node TBARS, FRAP and SOD values on the relapse free period of dogs with lymphoma

	Mean estimate	Median estimate	Median lower 95% CI	Median upper 95% CI		
LN-TBARS < 53 nmol/mg prot.	718.6	can not estimate	145	-		
LN-TBARS > 53 nmol/mg prot.	94.75	89	42	119	Log Rank	0.008
LN-FRAP < 6 µmol/mg	713.4	can not estimate	119	-		
LN-FRAP > 6 µmol/mg	101.25	89	42	129	Log Rank	0.0233
LN-SOD < 70 U/mg prot.	15	can not estimate	-	-		
LN-SOD > 70 U/mg prot.	104.8	119	42	129	Log Rank	0.006

EFFECTS OF CHEMOTHERAPY ON OXIDATIVE STRESS AND ANTIOXIDANT PARAMETERS

The "0" values are the basal untreated values when we started chemotherapy with doxorubicin. After one week, we could estimate the effect of the drug. According to the protocols two consecutive vincristine doses were given and after 2+3 weeks we could estimate the effect of vincristine. The complex effects of the chemotherapeutic drugs together with prednisolone, doxorubicin, vincristine and cyclophosphamide had been estimated in blood samples after after 2+3 weeks. After weeks 9 to 28 we could estimate the long-term cumulative effects of the drugs.

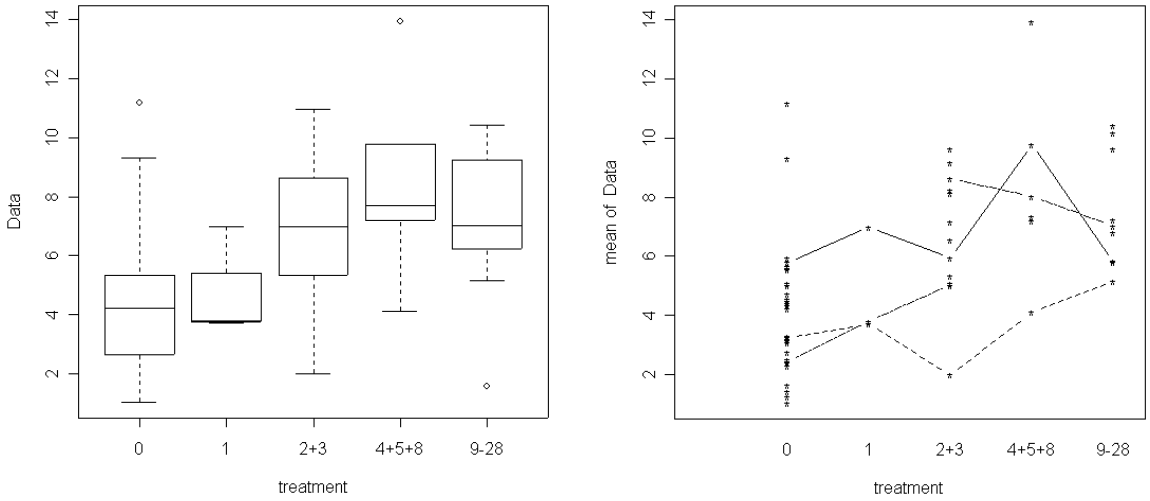
Plasma all trans retinol (mg/ml) concentrations showed a gradual increase during the chemotherapy (Table 9, Figure 8).

Table 9. Plasma-All-trans-retinol mg/ml values in dogs with lymphoma during treatment.

Weeks of treatment	Plasma-All-trans-retinol mg/ml	SD	Statistical similarities		
0	4.15	0.43	0	*	
1	5.89	1.28	1	*	*
2+3	7.04	0.63	2+3		*
9-28	7.20	0.68	4+5+8		*
4+5+8	8.57	0.93	9-28		*

ANOVA test showed statistical significance: $p<0.0001$

Figure 8. Changes of plasma-All-trans-retinol mg/ml values in dogs with lymphoma during treatment. a. Average and standard error of all dogs, b. All measured values of all dogs



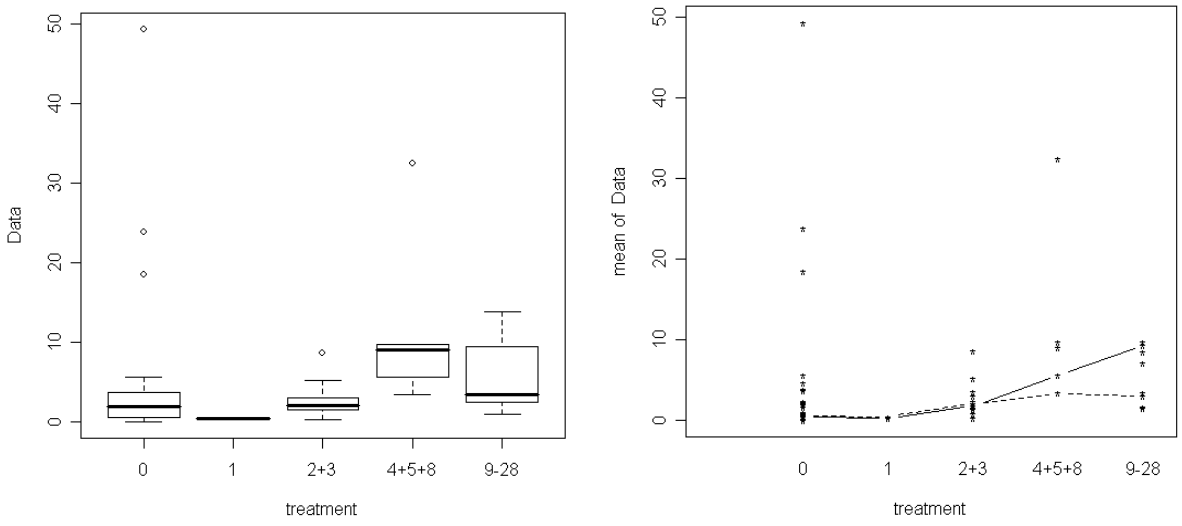
Plasma vitamin C concentrations (mg/ml) showed a gradual increase during chemotherapy (Table 10, Figure 9.).

Table 10. Plasma vitamin C concentrations (mg/ml) values in dogs with lymphoma during treatment.

Weeks of treatment	Plasma vitamin C mg/ml	SD	Statistical similarities		
0	4.29	1.68	0	*	
1	4.68	2.90	1	*	*
2+3	5.50	1.89	2+3	*	*
9-28	8.52	1.98	4+5+8		*
4+5+8	10.16	2.28	9-28		*

ANOVA test showed statistical significance: $p< 0.0053$

Figure 9. Changes of vitamin C concentrations (mg/ml) in dogs with lymphoma during treatment. a. Average and standard error of all dogs, b. All measured values of all dogs



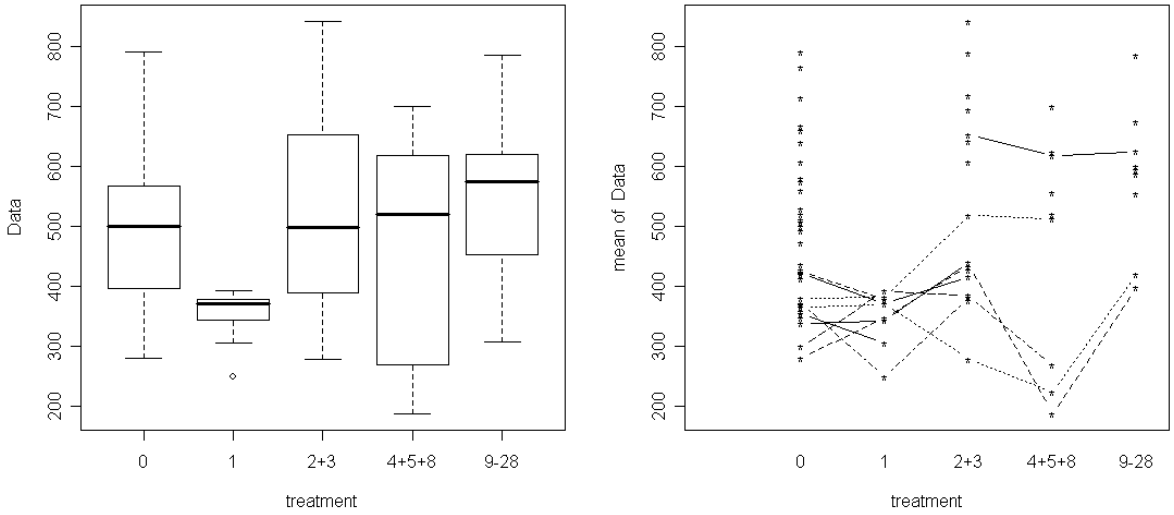
Red blood cell TBARS concentrations (nmol/g prot.) showed a gradual increase during chemotherapy (Table 11, Figure 10).

Table 11. Red blood cell TBARS concentrations (nmol/g prot.) in dogs with lymphoma during treatment

Weeks of treatment		SD	Statistical similarities		
0	473.35	31.04	0	*	
1	494.12	21.56	1	*	
2+3	556.63	25.41	2+3		*
4+5+8	462.59	31.15	4+5+8	*	
9-28	579.89	28.70	9-28		*

ANOVA test showed statistical significance: $p < 0.007068$

Figure 10. Changes of red blood cell RBC TBARS values (nmol/g prot.) in dogs with lymphoma during treatment. a. Average and standard error of all dogs, b. All measured values of all dogs



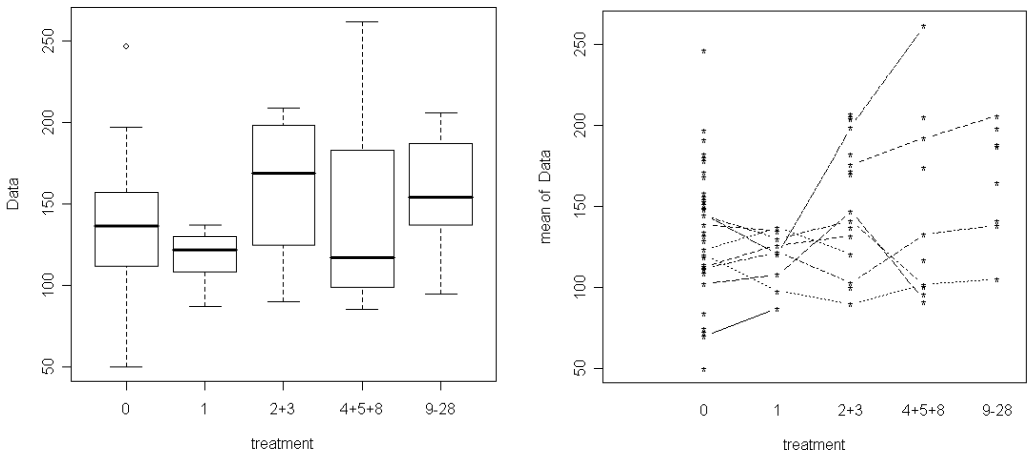
Red blood cell GSH-Px activities (U/g prot.) showed a gradual increase during chemotherapy (Table 12, Figure 11).

Table 12. Red blood cell GSH-Px activities (U/g prot.) in dogs with lymphoma during treatment

Weeks of treatment		SD	Statistical similarities		
0	135.73	6.33	0	*	
1	138.89	6.44	1	*	
2+3	146.68	8.42	2+3	*	*
4+5+8	150.51	6.65	4+5+8	*	*
9-28	133.45	8.93	9-28		*

ANOVA test showed statistical significance: $p < 0.03329$

Figure 11. Changes of red blood cell GSH-Px activities (U/g prot.) in dogs with lymphoma during treatment. a. Average and standard error of all dogs, b. All measured values of all dogs



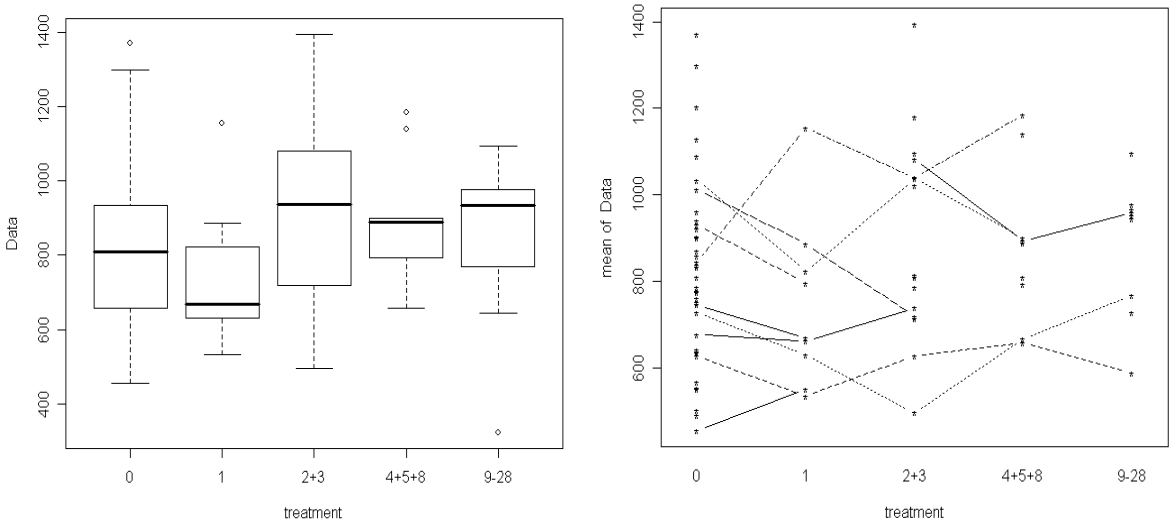
Red blood cell SOD activities (U/g prot.) showed a gradual increase during chemotherapy (Table 13, Figure 12).

Table 13. Red blood cell SOD activities (U/g prot.) in dogs with lymphoma during treatment

Weeks of treatment		SD	Statistical similarities		
0	762.27	34.26	1	*	
1	822.82	35.52	0	*	*
2+3	862.07	51.59	4+5+8		*
4+5+8	829.44	31.74	2+3		*
9-28	909.99	38.60	9-28		*

ANOVA test showed statistical significance: $p < 0.0053$

Figure 12. Red blood cell SOD activities (U/g prot.) in dogs with lymphoma during treatment. a. Average and standard error of all dogs, b. All measured values of all dogs



Discussion

AGE AND GENDER.

The average age of our patients was less (mean: 6.84 ±2.32, median: 7.01) than it has been recently published (median: 9.5 years/range: 4–14 years/),[45] although not different from other reports.[46,47,48] It is also known that female dogs are slightly overrepresented with this disease than males.[49] In this series, we had 1 more male case than females, but among treated patients one more female dog was included than males. Meanwhile, in this series we found that female dogs lived shorter but relapsed later than male dogs.

TREATMENT EFFICACY AND CLINICAL FINDINGS.

Our clinical results showed similarity to previous findings in stages, substages,[50] therapeutic response to CHOP and L-CHOP chemotherapy, and histopathological types and subtypes of canine lymphoma.[27] Although, a recent article reports higher survival values using a modified Madison-Wisconsin protocol. The treatment efficacy of our cases is comparable to other reports i.e Pioggi et al. (2017).[51] Our cases relapsed and died due to lymphoma with the median: 276 and 368 days, respectively. We found that the estimated median RFP and lymphoma-related survival time for all dogs in Pioggi at all's study was 414 days (95% confidence interval (CI), range 228–600 days) and 442 days (95% CI, range 236–648 days), respectively. Another study reported 121 cases treated by various chemotherapy, the median survival time (MST) for all 121 cases was 300 days (range, 1-1644 days).[52] Another article reported that median RFP was 196 days (range 22-1656 days) and OST was 292 days (range 40-2246 days) for all 104 dogs that received CHOP-based protocols. In our study, we observed similar RFPs as was reported earlier by others.[46,47] One of the most recent articles reported that the median PFS (progression-free survival) and median ST (survival time) in the L-asparaginase supplemented CHOP group were 344 days (range: 28–940 days) and 344 days (range: 70–940 days), respectively. The dogs that received CHOP without L-asparaginase showed a median PFS and median ST 234 days (range: 49–1822 days) and 314 days (range: 50–1822 days), respectively. The dogs that received L-CHOP chemotherapy had a significantly longer PFS than the dogs that received CHOP chemotherapy ($p = 0.001$). No significant difference was observed in ST between the L-CHOP and CHOP groups ($p = 0.131$).[45] We started with CHOP and continued with L-CHOP as a rescue due to the high price of L-asparaginase.

The most frequent adverse drug effect observed in our study was diarrhea, and revealed that diarrhea, anorexia and thrombocytopenia were the most serious side effects of treatment which is consistent with the findings of other authors.[53] In another report the most common adverse effects with chemotherapy were neutropenia (22%), vomiting (21%), diarrhea (20%) and inappetence (20%). Adverse chemotherapy effects may occur in about 20-25% of canine patients.[54]

IMMUNOPHENOTYPE.

The difference between high-grade T- and B-cell types in respect of OST and RFP values is well known. In the present report, higher median values were found (median OST: B-cell: 404 and T-cell: 202 days, respectively; RFP: 462 and 276 days, respectively). In a study it was found 55 days for T-cell lymphoma, 200 and 256 days for indolent and aggressive B-cell lymphoma, respectively).[53]

KI67%.

The average Ki67% (30.38 ±12.01) obtained in this study is similar to the 34.5% (± 16.8), 30.2 (± 10.8) values found in earlier studies.[55] Our cut-off value (≤ 20%) for OST corresponded to a previous report which stated that Ki-67% was non-significantly different between groups of dogs with lymphomas with less than 20% Ki-67-positive cells and with 40–60% Ki-67-positive cells.[50]

OXIDATIVE STRESS PARAMETERS MEASURED IN LYMPH NODES (LN), RED BLOOD CELL HEMOLYSATES (RBC) AND BLOOD PLASMA (P).

Our reference ranges are like the values of many reports, but different from some other due to the different methods used and the special patient's selection whereas we did not analyze healthy subjects, but dogs with lymphoma, only (Appendix).

Our recent findings agree with our previous article,[13] although the number of patients were much higher in this case which can explain some greater differences. The exception is in RBC-GSH-concentration, as in this report we express the values in mmol/ g prot., and not µmol/g prot. Our results on the antioxidant enzyme activities and antioxidant status measured in plasma and RBC-hemolysates in dogs with lymphoma show similarities to the findings of those who analyzed plasma antioxidants in leukemic humans.[26]

We found that plasma GSH values were higher in older dogs than in young ones, which is opposite to the result of authors, who stated that a significant correlation between the decreasing GSH concentration ($P = .028$; $r = -0.365$) and increasing age can be found in healthy dogs (Table 1).[56] They found that No significant correlation between age and plasma cysteine or Vitamin C concentrations were identified in their healthy dog population. A significant correlation of decreasing GSH concentration with increasing age was documented in healthy dogs. They did not find significant differences in antioxidant concentrations between healthy neutered male and spayed female dogs. Nevertheless, it does not contradict our findings, as we did not analyze healthy dogs, and we found these results in blood plasma and not in RBC hemolysates.

Authors found that sick dogs had significantly lower reduced GSH concentrations (median 1.22 mM), compared to healthy dogs (median 1.91 mM; $P = .0004$), and glutathione concentrations were significantly lower in dogs with severe disease relative to the mildly affected dogs, $P = 0.0379$.[56] This finding corresponds with our results, which show a decrease of plasma GSH levels in dogs with adverse drug reactions. At the same time, the antioxidant levels (all-trans retinol, Vitamin C in plasma and GSH-Px, SOD in RBC hemolysates) showed a gradual increase during chemotherapy with a decrease of tumor mass. In some articles it was found that affected dogs with decreased glutathione concentrations can present an increase in oxidative stress parameters including, 8-F2α-isoprostane26 and oxidized glutathione (GSSG),[57] or decreased antioxidant enzyme activity.[58] It can be suggested that decreased GSH concentrations may result from increased oxidative stress, or *vica versa* the low GSH concentration might sensitize the patients to oxidative stress.

It was found that FRAP values in lymph node were lower in those cases when Ki67% was higher (>30-40%), compared to lower Ki67% (5-30%). Although, in cases with very high Ki67% (> 40%), FRAP values varied a lot, and it is still possible that proliferation rate causes an increased utilization of the antioxidant factors of the tissues up to a limit when adjustment to oxidative stress results in increased FRAP levels (with high individual ranges) (Table 2).[13,59] It is still a question what the proper mechanism was when lymph node FRAP values were significantly higher than 6 µmol/mg and the relapse free period was lower than with FRAP < 6 µmol/mg (mean: 101.25 and 713.4, respectively) (Table 8).

OXIDATIVE STRESS, ANTIOXIDANT PARAMETERS AND THEIR IMPACT ON SURVIVAL.

Plasma GSH/GSSG values and plasma retinil palmitate levels showed a decrease with longer survival and longer relapse-free periods in dogs treated with chemotherapy (Table 3, 4, 5). This can be explained by the effects of chemotherapy which have an impact on oxidative stress mechanisms. It was stated that cancer cells generally present a higher oxidant level, which suggests a dual therapeutic strategy by regulating redox status (i.e., pro-oxidant therapy and/or antioxidant therapy). Therefore, pro-oxidant therapy exhibits a great anti-cancer capability, attributed to a higher oxidant accumulation within cancer cells.[60] Retinil palmitate is a derivative of vitamin A (retinol). As retinol is absorbed, it is metabolized into retinyl esters, mostly palmitate, in the enterocytes and secreted within chylomicrons into the lymphatic system.[61] It was found that retinyl palmitate combined with beta carotene increased the risk of lung cancer in smokers in a large CARET trial.[62] Moreover, it was found that higher risk of non-Hodgkin's lymphoma was associated with increased circulating retinol (OR = 0.90 [0.61-1.33]; Ptrend = 0.04).[63] So, there are some data which support our findings. However, we found that not plasma retinil palmitate, but higher all plasma trans-retinol levels were associated with longer survival (Table 8). These findings are contradictory, even though, articles state that the role of retinol levels and cancer risk is not fully understood. Authors of one of the most relevant articles on this topic state that the role of retinol in cancer risk might differ by organ site, with beneficial associations for lung and liver cancers, a harmful association for prostate cancer, and no association for cancer at other sites. Future studies exploring the role of retinoids in cancer at various sites would be useful in understanding the underlying mechanisms and clarifying the potential role of vitamin A in cancer etiology and prevention.[64]

Glutathione peroxydase activity was the lowest in lymph nodes of dogs treated with chemotherapy, with the longest survival (Table 6). This finding can be explained

by suggesting that antioxidant activity may be necessary to support cancer cells' viability and/or invasive capacity during tumor progression.[65] Not GSH-Px but lymph node glutathione S-transferase (GST) is known to have a greater impact on drug resistance in non-Hodgking's lymphoma, and studies reported in both human and canine lymphoma showed that genetic polymorphisms of GST has great importance in the treatment efficacy .[66,67] Nevertheless, it seems that GSH-Px plays some role in this mechanism, too. Glutathione peroxidase 4 (GPX4), an antioxidant enzyme, has biologically important functions such as signaling cell death by suppressing peroxidation of membrane phospholipids. It was found in human patients with diffuse large B-cell lymphoma that the GPX4-positive and 8-hydroxydeoxyguanosine-negative groups had a significantly worse prognosis than the other groups in both overall survival (P = 0.0170) and progression-free survival (P = 0.0005).[68] Other authors found that GPX1 gene expression was found to be significantly associated with early failure relapse of dogs with diffuse large B-cell lymphoma.[21] Authors suggest that high expression of GPX1, the major effector of GSH-dependent ROS defense, is adversely associated with early failure as well as disease-specific overall survival.

These suggestions are supported by our findings that low lymph node GSH-Px activities (cut off 16 U/mg prot.) were associated with longer survival. Median OST of dogs with GSH-Px lower and higher than 16 U/mg prot.: 510 and 49 days, respectively.

Concerning one of the oxidative stress parameters, TBARS measured in lymph nodes, we found that median survival (OST) for lower TBARS values than 53 nmol/mg prot. can not be estimated (mean: 756.13 days), but for higher than 53 nmol/mg prot. values 153 days were calculated (Table 7). Lymph node TBARS had a similar impact on the relapse-free survival, too. Other authors found that TBARS (malondialdehyde, MDA) in blood serum was higher in dogs with lymphoma compared to healthy subjects, and even higher in dogs after finishing chemotherapy.[22] These findings support the theory that oxidative stress promotes multiple oncogenic events, including cell proliferation, angiogenesis, migration, metabolic reprogramming, and evasion of regulated cell death in cancer cells. Moreover, oxidative stress plays a great role in the development of cancer by affecting genomic stability and signaling pathways within the cellular microenvironment. Elevated levels of ROS disrupt cellular homeostasis and contribute to the loss of normal cellular functions, which are associated with the initiation and progression of various types of cancer.[68]

Plasma vitamin A (all-trans retinol) levels in patients with lymphoma seemed to be also prognostic viewing the overall survival time, as lower than 4 mg/ml values were associated with 202, and higher than 4 mg/ml with 510 days, respectively (Table 7, Figure 7). Other authors reported that higher total serum carotenoids ($OR_{T3\ vs\ T1}$ = 0.66 [0.46-0.96]; P_{trend} = 0.02), were associated with a lower risk of non-Hodgkin's lymphoma. For retinol (OR = 0.90 [0.61-1.33]; Ptrend = 0.04), a statistically significant inverse linear trend was detected.[63] Although, other reports state that after multivariable adjustment, higher serum retinol was not associated with overall cancer risk (highest vs. lowest quintile: hazard ratio (HR) = 0.97, 95% confidence interval (CI): 0.91, 1.03; *P* for trend = 0.43).[64]

The relapse-free period was lower with high lymph node TBARS values, as mentioned before, just like with high FRAP values (Table 8). Lymph node SOD levels showed an opposite relationship. Low SOD levels were associated with low relapse-free period. High FRAP values with increased TBARS can be a consequence of longer term oxidative stress and as consequence increase antioxidant defense. Tumor cells develop a mechanism where they adjust to the high ROS by expressing elevated levels of antioxidant proteins to detoxify them while maintaining pro-tumorigenic signaling and resistance to apoptosis.[70] This can be the reason for the similar change in TBARS and FRAP levels and their effect on the relapse-free period. Similarly to serum TBARS, authors fond that serum FRAP values were also higher in dogs with lymphoma and even higher after finalizing chemotherapy.[22] It was also stated by other authors that 2 superoxide dismutases (MnSOD and EcSOD), glutathione peroxidase, and catalase gene expression in diffuse large B-cell lymphoma affected lymph nodes were all decreased in dogs with the worst outcome. Cu,ZnSOD expression showed no correlation with prognosis.[20] Although, we analyzed Cu,ZnSOD in lymph nodes and found association with low activity levels with worst prognosis.

Low SOD values can be a result of protein damage due to high ratio of oxidative stress. Meanwhile, redox protein alterations have been reported in several tumor types. Generally, antioxidant defense enzymes are decreased in tumor tissue when compared with normal tissue.[71,72] This is particularly true for MnSOD, which acts as a tumor suppressor in many model systems[71]. Recent data also implicate the up regulation of antioxidant defense enzymes in the tumor suppressive effect of BRCA1.[73,20] It can be suggested that less aggressive and non-resistant tumor cells, might have high SOD levels as this enzyme might act as a tumor suppressor.

Various parameters showed a gradual increase during chemotherapy, showing an improvement of general health state, such as plasma all-trans retinol and vitamin C concentrations (Table 9, 10). Concerning vitamin A, it was reported that vitamin A deficiency due to poor diet may be one of the contributing factors in cancer development.[74] Although, high levels increase the risk of breast cancer development.[75] It was reported that a significant number of patients with cancer had inadequate plasma ascorbate concentrations. Low plasma status was more prevalent in patients undergoing cancer therapy.[76] Authors found that in the dogs with lymphoma, a-tocopherol concentrations were higher and vitamin C levels were lower after treatment.[16] In contrast to these findings we did not find marked gradual elevation of alpha-tocopherol, but we found it with vitamin C.

CHANGES OF OXIDATIVE STRESS AND ANTIOXIDANT PARAMETERS DURING CHEMOTHERAPY

Red blood cell TBARS, GSHP-Px and SOD activities showed a gradual increase during chemotherapy, which can be either caused by an improved health state, or we may consider an upregulation of the synthesis of both

antioxidant enzymes due to the low rate and chronic oxidative stress effects caused by the chemotherapeutic drugs (Table 11, 12, 13).[77] Concerning SOD upregulation, authors suggested that tumor-associated inflammation mediates SOD-2 upregulation through NF-κB pathway, which may contribute to epithelial-mesenchyme transition and cell migration in AFG_1-induced lung adenocarcinoma.[78] Moreover, the authors showed oxidative stress and compensatory antioxidant enzyme upregulation in SOD1-G93A skeletal muscle.[79] The gradual increase of these antioxidant enzymes might be a good marker for the transient recovery of dogs with lymphoma.

Understanding and management of adverse drug reactions is a challenging mechanism in chemotherapy. Almost every chemotherapy drug can cause adverse drug reactions. Altogether 58.1 % of our cases had adverse effects. We suggest that these occasions were about glutathione depletion which is associated with augmentation of an oxidative stress-mediated pro-inflammatory state in a ROS-dependent mechanism. Meanwhile, the IkappaB-alpha/NF-kappaB pathway may be necessary for redox-mediated regulation of cytokines.[80] Patients are often advised to take antioxidants to decrease the severity of adverse drug reactions, and they might be beneficial against these events, meanwhile, antioxidants can interfere with the otherwise beneficial oxidative drug effects against tumors and contribute to drug resistance.

LIMITATIONS

There are several limitations to this study. There is a low number of cases, especially of those with the T-lymphoma. There was limited availability of enough data in some cases, as we could not get in contact with each owner after finishing the therapy. It would have been better to check each owner whether they supplemented the treated dogs with antioxidants without reporting it. Moreover, some measurements failed due to the difficulties of sample preparations and measurements, as these are not robust methods. We could not take blood samples from each treated dog during their treatment as many owners lived in the countryside and some treatment procedures were managed by their local treating vets.

Conclusions

In conclusion, we can state that oxidative stress mechanisms and the antioxidant system are very flexible in dogs with lymphoma, either by contributing to the development of cancer and its increased proliferation and resistance, or by showing alterations during cancer treatment. The proper mechanisms are unclear and the role of these parameters in prognosis is also questionable. We must mention that the individual alterations of the parameters are very wide. We tried to visualize it by adding figures about individual changes. We do not believe that these parameters could be used easily as prognostic indicators in a wider range of dogs, but that these findings can help in understanding the complexity of oxidative stress and antioxidant systems in dogs with lymphoma and their treatment. Some antioxidants show benefit (i.e. plasma retinil palmitate), some are not, like plasma GSH and GSH/GSSG with cancer therapy. Some show decrease during adverse effects (i.e. plasma GSH, some are not are not. High lymph node GSH-Px and high oxidative stress (TBARS) are associated with shorter survival, while high lymph node SOD and plasma all-trans retinol is associated with longer survival. The findings are explainable, although sometimes do not seem to be logical. The complexity of oxidative stress and antioxidants is marked with cancer and inflammation. While one interferes into cancer biology by therapy many processes are changing into an opposite side which also do not seem to be logical for the researcher (i.e. the oxidative stress effect of the drugs and the increase of antioxidant levels during the treatment). It seems that these processes are like flowing water. For instance, the direction of flow goes to the opposite when you throw a oace of stone into it. As Plato mentioned "Everything flows" (Greek: πάντα ρει) - Heraclitus, I believe, says that all things pass and nothing stays, and comparing existing things to the flow of a river, he says you could not step twice into the same river (Greek: δὶς ἐς τὸν αὐτὸν ποταμὸν οὐκ ἂν ἐμβαίης.). (Plato Cratylus 402a = A6).[81]

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82. Appendix
83. Reference intervals
84. Method: Box-Cox transformed data, Standard

Appendix

Lymph node		TBARS nmol/mg prot.	FRAP µmol/mg prot	SOD U/mg prot	GSH-Px U/mg prot
N		17	17	17	17
Mean		2,6	1,8	7,6	2,7
Median		2,5	1,9	7,4	2,7
SD		0,4	1,0	3,7	1,2
Mininum		1,6	-0,1	0,2	0,5
Maximum		3,3	3,6	16,2	5,1
Lower limit		23,7	0,7	17,4	3,4
Upper limit		696,8	62,7	502,3	80,8
90% CI for lower limit		20,4	0,3	16,8	2,8
		29,8	1,4	22,9	5,1
90% CI for upper limit		228,8	29,4	265,5	41,0
		2711,7	131,4	804,5	137,3

Blood plasma		Retinil-palmitate mg/ml	All-trans-retinol mg/ml	Vit-C mg/ml	DL-alpha-tocopherol mg/ml	FRAP µmol/l	GSH/ GSSG	GSSG µmol/l	GSH µmol/l
N		23	30	31	30	41	30	38	38
Mean		2,0	1,6	1,3	11,4	3,2	2,4	41050,0	223,0
Median		2,0	1,8	0,8	12,2	3,2	2,5	41050,0	248,1
SD		1,2	1,3	3,1	5,1	0,2	1,6	0,0	111,9
Mininum		-0,1	-0,9	-1,7	0,0	2,6	-0,4	41050,0	45,1
Maximum		4,5	4,8	13,2	21,0	3,6	5,2	41050,0	418,6
Lower limit		1,9	0,9	-	1,7	168,6	2,5	0,0	-
Upper limit		25,8	9,6	21,3	28,2	1300,7	70,3	229,1	1303,0
90% CI for lower limit		1,6	0,7	ND	ND	151,2	2,2	-107,3	ND
		2,5	1,4	ND	5,2	189,5	3,1	-60,6	94,8
90% CI for upper limit		17,4	7,8	8,4	24,6	921,3	41,7	171,9	1148,6
		36,3	11,4	37,6	31,9	1831,5	113,2	281,4	1423,4

Red blood cell hemolysate		GSH/ GSSG	GSSG µmol/g prot	GSH mmol/g prot	GH-Px U/g prot	TBARS nmol/g prot	SOD U/g prot
N		30	30	38	41	38	38
Mean		1,1	0,6	3,1	4,6	15,3	51,7
Median		1,0	0,5	3,1	4,6	15,2	51,4
SD		0,1	1,1	2,4	0,4	1,8	10,9
Mininum		0,8	-2,9	-1,3	3,6	9,5	30,4
Maximum		1,4	3,1	10,2	5,6	20,6	75,7
Lower limit		2,8	0,3	8,5	62,6	264,1	443,1
Upper limit		82,3	13,5	35,1	287,6	951,5	1327,1
90% CI for lower limit		2,5	0,3	ND	54,2	222,0	383,2
		3,4	0,5	8,9	72,3	310,0	511,5
90% CI for upper limit		24,5	8,0	29,2	240,7	824,3	1192,4
		ND	22,2	41,4	340,6	1077,5	1474,1