Summary of the Ph.D. thesis

INVESTIGATION OF PROGNOSTIC FACTORS IN CANINE NEOPLASTIC DISEASES

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1. Introduction and aims of the study

Lymphoma and mammary gland tumours are among the most common canine neoplastic diseases. Therapeutic resistance is an important clinical factor, especially in the case of tumours of lymphoid tissues, which usually leads to the failure of chemotherapy treatment. Although the treatment of mammary gland tumours is primarily surgical, adjuvant treatments would be beneficial, especially for high-grade advanced forms, but it is not yet possible as these tumours do not respond adequately to conventional cytostatic agents.

It has long been known that against certain types of tumours, cytostatic agents are not effective due to the ab ovo or developed resistance of tumour cells. Multidrug resistance (MDR) occurs when certain tissues or cells do not respond to the drugs used in the treatment. At present, MDR is one of the most important concerns in the treatment of cancer patients as it is mainly responsible for the failure of chemotherapy. However, several mechanisms of cellular MDR development are known, the most frequently observed form is due to the overexpression of P (permeability)-glycoprotein. It is the gene product of MDR-1, a 160-180 kDa ATP-dependent protein of the ATP-binding cassette (ABC) transporter superfamily. Pgp can release hydrophobic molecules, and also a significant amount of cytostatic agents to the extracellular space, which prevents drugs from reaching the required intracellular concentrations for antitumor activity. Among the most common agents used in canine chemotherapy protocols, Pgp has been proved to be efficient against vincristine, doxorubicin, actinomycin-D, mitoxantrone, etoposide, and vinblastine. Cross-resistance to these drugs has also been observed, which is a serious problem. Therefore, if resistance to any of the agents listed develops, tumour cells will also be resistant to other Pgp substrate

cytostatic agents, whether or not they have been used in the treatment before. Most dogs diagnosed with lymphoma relapse after the first cycle of chemotherapy treatment, and recurrent tumours are generally more resistant to cytostatic agents due to the presence of more resistant MDR clones.

Pgp overexpression is a long-known negative prognostic factor for lymphoma both in dogs and humans, with shorter relapse-free period and overall survival. The importance of determining the Pgp expression to serve as a prognostic factor before starting treatment has been demonstrated unequivocally by early studies of Bergmann (1996) and Lee (1996). However, later publications have found contradictory results, and explicitly different percentages of Pgp expression can be found in the literature. We hypothesize that these differences are partly due to methodological reasons, such as differences in the evaluation of staining, which comes from the subjectivity of investigators. Therefore, validation of various methods would be recommended to enhance the prognostic value. In this way, it would be possible to obtain information on the potential therapeutic resistance before starting treatment, and in these cases, non-Pgp substrates would be preferred over cheaper conventional chemotherapeutic agents.

Elevated Pgp expression was reported in treatment-resistant breast cancer in women. In case of dogs, only a limited number of studies have been available, however, these investigations found Pgp overexpression in cancerous breast tissue.

P53 tumour suppressor gene is the most frequently mutated gene in human cancers, which has been detected in more than 50% of human tumours. The protein encoded by the gene is a phosphoprotein located in the nucleus that is involved in the regulation of the cell cycle and the induction of apoptosis. It also plays an important role in the regulatory

mechanisms of cells that inhibit tumorigenesis induced by cancerous mutations by reducing the expression of proliferating cell nuclear antigen (PCNA) and MDR genes. Germ-line mutation of the p53 gene predisposes to various cancers, as the protein produced is inactive in this case. However, the expression of the protein in the cellular cytoplasm, or incidentally in the nucleus, often seems to be elevated, as the hapten used for the detection is identical in the mutant and intact protein. Germ-line mutation of the p53 gene has been described in both human and canine mammary gland tumours, and increased protein expression is considered as a negative prognostic factor.

Cyclooxygenases (COX, prostaglandin endoperoxide synthase) are key enzymes in the biosynthesis of prostaglandins. There are two isoenzymes: COX-1 and COX-2. The former plays an important role in homeostatic cellular functions, while COX-2 is involved in pathological processes such as inflammation, hyperalgesia, and tumorigenesis. It plays a vital role in the formation of tumour vasculature, tumour growth, and as the degree of malignancy increases, expression of COX-2 increases as well. In the case of several tumours (e.g., human colorectal tumours, cervical cancer, canine mammary gland tumours), there is a significant correlation between the elevated expression of COX-2 and the development of distant metastases. This results in shorter survival times, therefore, elevated levels of COX-2 can be considered as a negative prognostic factor in certain tumours, the inhibition of which may have clinical benefits.

Increased COX-2 expression can be detected in 40-50% of human breast tumours, and the inverse association between the incidence of breast cancer and the use of non-steroidal anti-inflammatory drugs (NSAIDs) in women has long been known. As in humans, healthy canine breast tissue shows no or minimal COX-2 expression. Contrarily, in most

mammary gland tumours, elevated COX-2 expression can be detected, which is, as a negative prognostic factor, associated with the degree of malignancy, relapse susceptibility, and metastatic potential. Several studies detected increased expression of COX-2 in canine malignant mammary gland tumours, with particularly high values in the more aggressive anaplastic forms. In recent years, an increasing number of investigations have been published, which confirmed the therapeutic benefit of using COX-2 inhibitors in high-grade mammary gland tumours in dogs.

Tyrosine kinases are specific signalling molecules that play a vital role in the regulation of cell growth and differentiation. Their receptors are located on the surface of cells, which can send signals to the nucleus by initiating phosphorylation cascades. They play an important role in the neovascularization induced by the tumour, which leads to the formation of the tumour's own vasculature. The altered function of kinases can be caused by a variety of processes, including mutations, increased expressions, fusion proteins, and autocrine loops. Mutations (e.g., point mutation, deletion, internal tandem duplication) can also cause the phosphorylation of protein kinases, even in the absence of a trigger signal. Among other things, these processes lead to increased cell growth and survival. In several human tumours, activation of abnormal signalling pathways due to dysregulation of tyrosine kinases is a known phenomenon, however, little information has been available so far in the case of canine mammary gland tumours. Dysregulation and over-expression of certain receptor tyrosine kinases have long been proved in human breast tumours. The best-known is HER-2 (ErbB2), which is involved in the process of tumorigenesis, and also found in 20-30% of breast cancers where, as a negative prognostic factor, indicates a shorter

survival time. Targeted inhibition of HER-2, VEGF, and EGFR are used as part of routine treatment.

1.2. Purposes of the study

1. Research aims in the case of canine lymphoma: Our main goal was to confirm the prognostic value of Pgp immunohistochemistry. In this context, we also aimed to validate our staining method by determining a Pgp cut-off value. A wide variation of percentages of Pgp expressions are reported in the literature, therefore, it is recommended to validate different methods to enhance the prognostic value. Our long-term goal was to obtain information on potential therapeutic resistance before starting treatment, to develop a more personalized treatment.

Another aim of our study was to evaluate various clinical factors, mainly focusing on mortality due to relapses and side effects of drugs, and to identify the related prognostic factors, and investigate their relationship with MDR.

2. Research aims in the case of canine mammary gland tumours: Our main goals were to investigate the causes of therapeutic resistance, determine the prognostic values of several factors observed in human oncology, as well as to identify potential novel therapeutic targets (presentation of selective COX-2 inhibitors approved for use in dogs, small molecule tyrosine kinase inhibitors).

When investigating Pgp expression in canine mammary gland tumours, we aimed to confirm its role in the development of MDR and evaluate its prognostic value. P53 expression was also examined in these samples. We also wanted to confirm the prognostic value of p53 and investigate correlations between Pgp and p53 expression.

In the case of mammary gland tumours, one of our main goals was to perform the immunohistochemical investigation of COX-2 expression, to confirm its prognostic value, and also demonstrate and compare clinical benefits from the use of various COX inhibitors.

Another goal was to investigate tyrosine kinases which are involved in tumorigenesis in the case of canine mammary gland tumours, and thereby identify novel therapeutic targets. The following tyrosine kinases and VEGF were detected by real-time PCR: VEGFR1, VEGFR2, EGFR, ErbB2 (HER-2), PDGFRα, KIT, and MET. Most tyrosine kinases investigated in our study are targets of masitinib and toceranib, which are small molecule tyrosine kinase inhibitors registered in Europe for use in dogs.

2. Materials and methods

2.1. Investigation of Pgp expression in canine lymphoma

Our study involved 33 dogs of different breeds and ages, 18 males and 15 females, their average age was 7.36 years.

At the first examination, patients were classified into stages: Stage II (n=1), Stage III (n=4), Stage IV (n=22) and Stage V (n=6). Histological evaluation of lymph node samples lead to the diagnosis of high-grade lymphoma in 26 (78.78%) dogs (3 B-lymphoblastic, 13 diffuse large B-cell with high mitotic index, 8 peripheral T-cell, and 2 Burkitt-like lymphomas), and low-grade lymphoma in 7 (21.21%) dogs (5 diffuse large B-cell centroblastic lymphomas with low mitotic index, 1 Mantle-cell, and 1 T-zone lymphoma).

Patients were treated with COPA and Madison-Wisconsin (MW) protocols. 10 dogs received both protocols (COPA first, MW after relapse).

Adverse reactions during treatments were classified based on the recommendation of the Veterinary Cooperative Oncology Group from 2011. The classification was performed according to the severity (grade) of side effects, and quantification was also carried out on this basis. In addition, cumulative scores were calculated based on the number and severity of adverse reaction episodes.

Immunohistochemical detection of Pgp was based on the article published by P. E. Ginn in the Veterinary Pathology Journal in 1996, and also on the recommendation of associates of the National Institute of Oncology. Samples were deparaffinised and rehydrated in xylene, followed by immersion in descending alcohol series. Endogenous peroxidases were blocked by using 3% hydrogen peroxide solution. Following antigen retrieval and repeated washing with PBS, non-immune origin bindings were blocked with normal horse serum. C494 mouse monoclonal antibody was used to react with Pgp, then the immunohistochemical reaction was visualized by a streptavidin-biotin immunoperoxidase system (Vectastain ABC-kit) and diaminobenzidine (DAB). Contrast staining was performed with Mayer's haematoxylin.

Intact canine liver and kidney tissues were used as positive controls, while healthy lymph node tissue was used as negative control. In addition, an 'internal negative control' was applied, where the primary monoclonal antibody was replaced with PBS.

The degree of staining of the cells and its intracellular localization were evaluated by using a light microscope, with a double-blind method performed by two experienced investigators. The degree of Pgp expression was marked on a 4-point scale based on an estimate of the number of positive cells. The intensity of staining was evaluated on a 4-point scale irrespectively of the Pgp expression scoring system.

Median relapse-free period (RFP) and overall survival time (OST) were determined by Kaplan-Meier analysis. Relapse-free period and overall survival time of different groups were compared using the log-rank test and Cox proportional hazard analysis. Fisher's exact test was performed to determine correlations between different variables, where results were considered as significant at $p<0.05$. Specificity and sensitivity values were calculated using ROC analysis to determine the Pgp expression cut-off value. Two sample Student's t-test with unequal variances was applied to compare clinical and laboratory parameters of different groups. Tests were performed using Statistica, Microsoft Excel, and R Companion. The results obtained were considered significant at $p<0.05$.

2.2. Investigation of Pgp and p53 expression in canine mammary gland tumours

Our study included 30 dogs of different breeds and ages diagnosed with mammary gland tumours. Routine histological examination of the tumours resulted in 9 tubulopapillary carcinomas, 12 complex carcinomas, 2 complex adenomas, 2 benign mixed tumours, 1 fibroadenoma, 1 ductal papilloma, 2 carcinosarcomas, and 1 lipid-rich complex carcinoma.

Sections were initially fixed in 4% neutral formalin solution, then embedded in paraffin. Immunohistochemical detection of p53 was performed according to the publication of Wolf et al. in 1997. Samples were deparaffinised and rehydrated with the same method as in the case of Pgp detection. Following antigen retrieval and repeated washing with PBS, non-immune origin bindings were blocked with normal horse serum. CM-1 rabbit primary polyclonal antibody (Signet Laboratories) was used, and immunohistochemical reaction was visualized using a streptavidin-biotin immunoperoxidase system (Vectastain ABC-kit) and

diaminobenzidine (DAB). Contrast staining was performed with Mayer's haematoxylin.

Human breast adenocarcinoma was used as positive control, and healthy canine lymph node as negative control.

The staining intensity of cells was examined with a light microscope using a double-blind method performed by two experienced investigators. The level of p53 expression was marked on a 4-point scale based on an estimate of the number of positive cells. Cytoplasmatic and nuclear staining were not evaluated separately, as nuclear staining could not be observed in 56% of the samples.

Patients were divided into 4 groups based on the histopathological classification of the tumours: tubulopapillary carcinomas (n=9), complex carcinomas (n=12), other malignant tumours (n=3), benign tumours (n=6). In each group, we examined whether neutering had occurred prior to the onset of the mammary gland tumour, the age of the patients at the time of surgical excision of their mammary gland tumour, whether the tumour that we investigated was a primary or recurrent tumour, the presence of metastatic lymph nodes or invasiveness, the onset of recurrence or other neoplastic diseases before or after the surgical excision of the tumour, and whether the decease of the animal was caused by cancer.

Differences among various parameters of the groups diagnosed with different tumours were examined by performing the Wilcoxon rank-sum test and the two-sample Student's t-test with unequal variances, using SAS-STAT software. Among the parameters, Pearson's correlation analysis was performed with the SPSS 8.0 computer software. The results obtained were considered as significant at $p<0.05$.

2.3. Investigation of COX-2 expression in canine mammary gland tumours

In our study, mammary gland tumour samples from 42 dogs of different breeds (40 females, 2 males) were examined. The average age of patients was 9.17 years.

Following surgery, 31 patients received NSAID treatment for at least 4 months, including 16 patients who received multiple agents: piroxicam (n=23), meloxicam (n=18), firocoxib (n=12), which is due to the long duration of the study and the subsequent presentation of advanced agents.

Altogether 13 dogs received chemotherapy treatment postoperatively, 4 of them were treated with multiple agents: doxorubicin (n=7), cyclophosphamide (n=1), carboplatin (n=10).

Paraffin-embedded sections were used for immunohistochemical tests. The samples from surgically removed tumours were fixed in 4% neutral formalin solution. Immunohistochemical detection of COX-2 expression was performed according to the publication of Queiroga et al. in 2007, and the recommendation of associates of the National Institute of Oncology. Samples were deparaffinised, rehydrated, and non-immune origin bindings were blocked the same way as described for Pgp detection. Samples were then incubated with monoclonal primary IgG1 (Rat Cox-2 aa. 368-604) antibody. Further process of the staining method was performed the same way as described for Pgp detection. Positive controls were healthy canine liver and kidney tissue samples, similarly to the study of Queiroga et al.

The staining was evaluated with a light microscope using a double-blind method performed by two experienced investigators. In addition to the calculation of the percentage of COX-2 expression, the intensity of

staining was also determined on a 4-point scale by estimating the number of positive cells and the intensity of coloration.

28 tumour samples were from primary, while 15 samples were collected from recurrent tumours (1 patient had both primary and recurrent tumours). Patients were divided into 5 groups based on histopathologic classification of the tumours: benign (n=13), simplex and anaplastic simplex carcinoma (n=9), complex carcinoma (n=8), tubulopapillary carcinoma (n=8), and carcinosarcoma (n=4). Based on histologic Grade, the distribution of malignant tumours was the following: Grade I (n=14), Grade II (n=7), Grade III (n=8). The result of the stage classification in the case of benign tumours was the following: Stage I (n=10), Stage II (n=3), Stage III (n=0), Stage IV (n=0). In the case of malignant tumours: Stage I (n=8), Stage II (n=9), Stage III (n=7), Stage IV (n=5).

COX-2 expression values were compared among different groups, and correlation between COX-2 expression and tumour size, and various parameters related to survival was investigated. Kruskal-Wallis ANOVA and Bonferroni tests were performed. Relapse-free period and overall survival time of different groups were compared using log-rank test and Cox proportional hazard analysis. Specificity and sensitivity values were calculated using ROC analysis when determining the COX-2 expression cut-off value. Statistical analyses were performed using SPSS 8.0, Free Statistics Software (WESSA, 2013), Microsoft Excel 7.0, and StatsDirect 3.2.8. softwares. The results obtained were considered as significant at $p<0.05$.

2.4. Investigation of the effect of tyrosine kinases in canine mammary gland tumours

Our study included 13 female dogs with mammary gland tumours, whose average age was 9.8 years. Histological classification of the

tumours was simplex carcinoma (n=5), complex carcinoma (n=5), spindle cell carcinoma (n=1), osteosarcoma (n=1), and chondrosarcoma (n=1). In each case, a healthy teat was also removed surgically that did not show any cancerous lesions, and served as a control. The aim of our study was to detect the following tyrosine kinases and VEGF in canine mammary gland tumours using real-time PCR: VEGFR1, VEGFR2, EGFR, ErbB2 (HER-2), PDGFRα, KIT, and MET.

In our study, we used teats that did not show any signs of necrosis. Samples of 0.5 to 1.0 g were removed and homogenized with Trizol reagent, until further processing they were stored at -80ºC. Following RNA extraction and DNase digestion, cDNA was synthesized using RevertAidTM H Minus First Strand cDNA Synthesis Kit (Fermentas). IQTM SYBR Green Supermix Kit (Bio-Rad) was used for real-time PCR measurements. Primers were designed with Primer-BLAST tool, and ordered from Sigma-Aldrich and Bio-Science. Two parallels were set from each sample in which we examined the mRNA of nine proteins.

PCR measurements started with an enzyme activation at 95ºC for 3 minutes. This was followed by 40 cycles in this order: 95ºC for 10 sec, 56ºC for 10 sec, and 72 º C for 20 sec. Final products of the reactions were verified by running an agarose gel, and also by using a melting curve analysis.

Expression values of cancerous and healthy teats were compared for each patient, and patients were divided into groups according to the histologic degree of malignancy: Grade I (n=5), Grade II (n=5), and Grade III (n=3). Student’s t-test, Kruskal-Wallis ANOVA, and Mann-Whitney tests were performed with Statistica software. The results obtained were considered as significant at $p<0.05$.

3. Results and discussion

3.1. Investigation of Pgp expression in canine lymphoma

In our samples, the average degree of Pgp expression was 34.2%. Of all the patients examined in our study, 54.5% was found to be Pgp-positive, which is considered as a high percentage according to literature data. Pgp staining values detected by immunohistochemical method show a high deviation in dogs. Different results may occur due to several factors, such as methodological differences (strength of antigen retrieval, evaluation methods, etc.), a variety of patients or different chemotherapy protocols. However, in the case of our study, the higher value can be explained by the relatively high number of T-cell lymphoma patients (27.7%). Therefore, it is necessary to define cut-off values and validate methods.

When using the scoring system, significant differences were found in overall survival time and relapse-free period between groups with different scores. Fifteen patients were considered as Pgp negative (0 point), while 18 were Pgp positive (1, 2, 3 points), this ratio is consistent with literature data. Significant differences were found between the Pgp positive and negative groups in overall survival time (240 days vs. 428 days, $p=0.0027$) and relapse-free period (95 days vs. 232 days, $p=0.004$). ROC analysis was used to define a cut-off value for Pgp expression, which was 35% in this case, and its specificity and sensitivity for survival were 0.75 and 0.7, while 1 and 0.533 for predicting recurrence. According to this, Pgp immunohistochemistry possesses an adequate diagnostic value when using a cut-off value of 35%.

One of our goals was to investigate the intensity of staining and to quantify it. A significant difference (240 days vs. 428 days, $p=0.016$) was found in overall survival between strongly and poorly stained samples. A

similar trend was observed when comparing relapse-free period: 103 days vs. 221 days (p=0.046). The prognostic value of the intensity of Pgp staining was confirmed by our study. In addition, an increased intensity was observed in the case of T-cell lymphoma samples (8/9), which also explains their therapeutic resistance.

Summarizing the prognostic value of Pgp immunohistochemistry, the relationship between the expression of ABC transporters and the clinical course of diseases is contradictory. Although the importance of Pgp determination (as a prognostic factor) has been demonstrated in early studies (Bergmann, 1996; Lee, 1996), in 2015 Zandvliet et al. found that the levels of ABC transporter gene expression tested in pre-treatment samples were not predictive of overall survival time or relapse-free period. Dhaliwal et al. (2013) found a difference in the median survival time of Pgp negative and Pgp positive groups when using an immunohistochemical method, however, the difference was not significant. Despite these assumptions, we found that Pgp expression tested before treatment, with the use of a proper cut-off value, is a reliable prognostic factor. Prior to the study, we hypothesized that a cut-off value of 10% would have a good prognostic value. However, during the investigation, we concluded that using a higher cut-off value can lead to a more significant difference in survival.

One of the aims of our study was to compare the side effects of drugs with different Pgp expression values. The most common side effect observed in our study was diarrhoea. Cumulative grading showed that diarrhoea, anorexia, and thrombocytopenia were the most serious side effects of the treatment, as reported in the literature. We have found that cancer mortality, as well as mortality due to side effects of drugs, can be poorly predicted with the use of ≥6.5% Pgp cut-off value.

In our study, we also investigated whether normal drug resistance function in vivo can be inferred from pre-treatment tumour samples. According to our results, it is possible to predict mortality due to side effects of drugs with low sensitivity (0.55), and high specificity (0.81). Contrarily, mortality due to cancer could be predicted with high sensitivity (0.87) and low specificity (0.66). According to our experience, side effects of drugs occur more often in Pgp-negative than in Pgp-positive patients, and a remarkable significance ($p=0.001$) was observed in the onset of diarrhoea when using ≥6.5% Pgp cut-off value. This may be due to the fact that Pgp-negative patients survive longer, and therefore receive more chemotherapy treatment cycles, and thus have a greater chance of developing diarrhoea. We found that the onset of side effects has a weak correspondence with Pgp expression of pre-treatment tumour samples. Evaluation of Pgp expression provides relevant information on the therapeutic resistance of tumour cells, but not on the therapeutic response of the animal as a whole (especially drug side effects).

The low number of cases was the most important limiting factor of our study. Our results showed that pre-treatment Pgp-negative lymph nodes have higher OST and RFP values, which is consistent with literature data to the effect that in the case of canine lymphoma, elevated Pgp expression is associated with the development of therapeutic resistance, thus a negative prognostic factor.

Based on our results, the intensity of staining should also be evaluated when using immunohistochemical methods. It is assumed that different results obtained by researchers were not only due to various immunohistochemical methods or methodological differences, but also because of the higher prognostic value of Pgp expression in patients treated mainly with Pgp substrates.

In our study, we found that Pgp immunohistochemistry has an appropriate diagnostic value and can serve as a base for individualized treatment in canine lymphoma. In the case of intensive and high (>35%) Pgp expression, it is recommended for the treating veterinarian to select agents that are not Pgp substrates (e.g., lomustine, L-asparaginase) to achieve a better therapeutic response.

3.2. Investigation of Pgp and p53 expression in canine mammary gland tumours

Regarding Pgp expression, our results were consistent with literature data: 66.94 % in tubulopapillary carcinomas, 35.72 % in complex carcinomas, 25% in benign tumours, and 33.33% in carcinosarcomas.

The comparison of Pgp expression between tubulopapillary carcinomas and complex carcinomas revealed a significant difference, as well as in the case of malignant and benign tumours. This raises the importance of the fact that all types of malignant mammary gland tumours examined in our study, especially tubulopapillary carcinomas, can be considered as highly treatment-resistant tumours. It makes much less sense to treat these tumours with chemotherapeutic agents that are Pgp substrates (doxorubicin, vincristine, cyclophosphamide, mitoxantrone, etc.). These findings are consistent with clinical experiences that canine mammary gland tumours are resistant to most intravenous cytostatics (including Pgp substrates).

Pearson’s correlation test was used to examine our results: we found a negative correlation between Pgp expression and survival time, and a positive correlation between Pgp expression and relapse susceptibility, regardless of tumour histology. Therefore, the determination of Pgp expression in mammary gland tumours can be a good prognostic

parameter, regardless of whether these patients are treated with chemotherapy or with surgery alone.

In the case of p53 expression, positive staining was observed even in the case of benign tumours (43.3%), however, with lower expression than in tubulopapillary (85.6%) or complex carcinomas (66.5%). Significant differences were found in the expression values of tubulopapillary and complex carcinomas.

Pearson's correlation test showed that expression levels of Pgp and p53 are related, which means that increased expression of proteins that can cause therapeutic resistance and mutated p53 can similarly indicate a worse prognosis. However, a significant correlation could not be observed between p53 expression levels and survival.

Wilcoxon log-rank test was applied to compare median survival times between groups with different Pgp expression scores. In the group with lower scores (0, 1, 2 points; n=25), OST was 766 days, while in the group with a higher score (3 points; n=5), OST was only 36.5 days, and the difference was significant (p=0.0302).

Wilcoxon log-rank test was applied to compare median survival times between groups with different p53 expression scores. In the group with lower scores (0, 1, 2 points; n=22), OST was 803 days, while in the group with a higher score (3 points; n=8), OST was only 365 days, but the difference was significant. The lack of predictive value of p53 immunohistochemistry was later confirmed by other studies.

3.3. Investigation of COX-2 expression in canine mammary gland tumours

Our main goal was to investigate COX-2 expression in canine mammary gland tumours, and to confirm and compare clinical benefits of using various COX inhibitors. We hypothesized that elevated COX-2

expression is a negative prognostic factor in the case of dogs, too, and inhibition of which leads to a survival benefit.

According to our samples, malignant tumours were 69.05% of all cancers, which is higher than the usual rate of about 50% reported in the literature. The relatively higher number of malignant tumours may be due to the lack of early neutering, or because of the fact that patients are usually referred to oncologists later, when they are already in an advanced stage, thus malignant transformation of initially benign lesions is more likely to occur.

The results of our study were consistent with literature data: COX-2 expression of malignant mammary gland tumours is higher than that of benign tumours. When examining expression values in percentage, significant differences were found between benign tumours (21.96%) and tubulopapillary carcinomas (49.16%), benign tumours and simplex carcinomas (47.22%), and also between benign tumours and sarcomas (71.5%).

When investigating the intensity of staining using the 4-point (0-3) scale, significant differences were found between benign tumours (0.61) and simplex (anaplastic) carcinomas (1.72), benign tumours and tubulopapillary carcinomas (1.33), benign tumours and sarcomas (1.87), simplex carcinomas and complex carcinomas (1.00), and also in the intensity of coloration between carcinomas and sarcomas. These results are also consistent with literature data.

In the case of malignant mammary gland tumours, the median survival time was the following: 181 days for simplex carcinomas, 178 days for complex carcinomas, 190 days for tubulopapillary carcinomas, and 152 days for sarcomas.

We could not find a significant difference in the degree or intensity of COX-2 expression in tumours of different Grades, only a growing trend

was found with the increase of the degree of malignancy. These results are consistent with the literature, according to which particularly high expression values can be observed in the case of more aggressive, anaplastic forms.

Significant differences were not found in the degree and intensity of COX-2 expression in Stage I (under 3 cm in diameter), Stage II (3-5 cm in diameter), Stage III (over 5 cm in diameter), or Stage IV (any size with a metastatic lymph node) tumours.

We found a positive correlation between the level of COX-2 expression and the size of the tumour. According to literature data, tumours over 5 cm in diameter are more likely to metastasize than smaller tumours, and patients are 7x more likely to die within 2 years after surgery. An explanation for the biological characteristics listed here may be the higher COX-2 expression associated with larger tumour size.

In the case of malignant tumours, a negative correlation was found between the level of COX-2 expression and survival time, as well as relapse-free period when performing Pearson's correlation test. These data are consistent with the literature, to the effect that elevated COX-2 expression is a negative prognostic factor.

Log-rank test was used to compare the overall survival time (OST) of groups with different COX-2 expression scores. We found a significant difference ($p=0.002$): patients with higher scores (3 and 4) lived significantly shorter than those with lower scores (0, 1, and 2).

A cut-off value of 50% was determined to increase the prognostic value of COX-2 expression. In the case of malignant tumours, with the use of a 50% cut-off value, a significant and remarkable difference was found in survival time and relapse-free period. The 50% cut-off value, referred to about 1-year survival, had a low sensitivity (0.357), but high specificity (1), when referred to a 3 months long relapse-free period, it showed high

sensitivity (0.928), but low specificity (0.333). The overall survival time of the group with an expression value of >50% was 190 days, while it was 330 days for the group with an expression value of <50% (p=0.009). The relapse-free period was 110 days for the group with an expression value of >50%, while it was 300 days for the group with an expression value of <50% (p=0.039).

A particularly strong negative correlation was found between Grade classification and survival time (p<0.0001), and also between Grade classification and relapse-free period (p<0.0001). These results are consistent with the literature, to the effect that grading has a specifically strong prognostic value.

When comparing the overall survival time and relapse-free period of different stages, we found that there is no significant difference between Stage I, II and III, however, survival time of Stage IV patients was significantly shorter (p<0.0001). Our results were consistent with the experiences reported in other publications (Rasotto et al., 2017), which also showed that Stage IV patients with metastatic lymph nodes have a significantly shorter survival time than patients of other stages.

Chemopreventive and antitumor effects of NSAIDs have long been known, as well as the inverse correspondence between use of NSAIDs and risk of developing breast cancer in women. NSAIDs are part of the treatment for many types of tumours, although the exact cellular and biochemical mechanisms of antitumor activity are only partially known. With the increasing spread of metronomic protocols, the use of NSAIDs is gaining ground in clinical oncology, especially in the case of tumours that are resistant to intravenous chemotherapy. Metronomic protocols aim to inhibit the angiogenesis of tumours by a continuous, low-dose administration of COX-2 inhibitors or other chemotherapeutic agents, depending on the protocol.

In our study, patients treated with NSAIDs did not respond well: the group receiving NSAID treatment had shorter survival time. A possible reason for this result is the inadequate experimental design, since a greater proportion of dogs with more advanced and more aggressive tumours received treatment, while the control group consisted predominantly of patients with Grade I tumours who did not require adjuvant treatment. Of the agents used, firocoxib was the only one that correlated positively with survival time. The reason for this is probably the fact that firocoxib was the only selective COX-2 inhibitor tested in our study. Although similarly to other studies (Arenas et al., 2017), we could partially demonstrate the efficacy of firocoxib in the adjuvant treatment of high-grade canine mammary gland tumours, our results are limited by low patient numbers, inadequate patient selection and experimental design, as well as difficulty of follow-up. A differently designed experiment (e.g., patients with tumours of the same stage and grade) would have been necessary to achieve more relevant results, however, the focus was on COX-2 expression in our study.

In our study, patients receiving chemotherapy treatment did not respond well (OST was 224 days of the chemotherapy group compared to 246 days of the control group), the relapse-free period of the group that received cytostatic treatment was also shorter than that of the group that did not receive chemotherapy treatment. These results are consistent with previous studies on the chemotherapeutic resistance of mammary gland tumours. The shorter relapse-free period and overall survival time of the group that received cytostatic treatment can be explained by the fact that patients in this group had higher stage and grade tumours, while the control group consisted predominantly of patients with Grade I tumours, which indicates a more favourable prognosis, thus adjuvant treatment was not reasonable. The tendency was reversed when examining the overall

survival time of patients with Grade II and Grade III tumours: OST of the group that received chemotherapy remained 224 days, but that of the control group decreased to 152 days. Our results are very similar to those of Arenas et al. (2016), where the survival time of the group that received cytostatic treatment (mitoxantrone) was increased compared to the untreated group, but similarly to us, they did not experience a significant difference.

It should be noted that the efficacy of treatments cannot be appropriately studied in a relatively small (n=42) and heterogeneous population with several variables. However, we did not aim to provide reliable statistical analyses, as for such a study, patients of different treatment groups should be in the same or similar stage, have same or similar tumour malignancy, and age. Unfortunately, we did not have the opportunity to do such an investigation.

In general, COX-2 staining is not a tumour-specific characteristic, and is difficult to standardize. Previous publications have also reported huge differences in the staining of samples, which significantly impairs the diagnostic usefulness of this marker. However, increased COX-2 expression is an important biological characteristic that may be associated with malignancy and spread of the tumour. It is no coincidence that we found a significant difference in survival times with high (50%) cut-off values.

Our study successfully confirmed our initial hypotheses: similarly to previous studies, we were able to detect the elevated expression of COX-2 in advanced, large canine mammary gland tumours, and we found an unequivocal correlation between elevated expression and tumour size. We also observed an association between the tendency of elevated expression and poorer clinical outcomes (decreased overall survival time and relapse-free period), and degree of malignancy. These observations

are consistent with previous literature data, that COX-2 plays a vital role in tumorigenesis, tumour growth, degree of malignancy, and increases the risk of metastasis and relapse. As a result, NSAIDs (especially selective COX-2 inhibitors) may play an important role in the adjuvant therapy for canine malignant mammary gland tumours in the future. This is particularly true for high-grade forms with elevated COX-2 expression. The latter was also confirmed in our studies by affirming the positive therapeutic effect of firocoxib. This is especially important in the case of chemotherapy-resistant mammary gland tumours due to the lack of effective adjuvant treatment options so far.

In our study, we found that COX-2 immunohistochemistry has a high prognostic value, and it can be used as the basis of individualized medicine in the case of canine mammary gland tumours. In case of intensive and high expression values (>50% in our case), the treating veterinarian should definitely recommend the use of a COX-2 inhibitor after the surgical excision of the tumour, since firocoxib has been proved to be effective of the available agents.

3.4. Investigation of the effect of tyrosine kinases in canine mammary gland tumours

We found that all of the listed factors were present in significantly higher amounts in the cancerous mammary gland tissue than in the healthy teat of the animal. Higher-grade tumours were associated with significantly higher VEGFR1, VEGF, c-KIT, and c-MET expression. Grade I tumours showed the highest mRNA levels of VEGFR2, PDGFR1, EGFR, and ErbB2 expression, followed by Grade II, and Grade III tumours with decreasing values.

Relative expression values of the groups with different grade tumours were compared to their healthy control teat pairs. In the case of Grade III tumours, VEGFR1, c-KIT, and c-MET relative expression values were significantly higher than in Grade I and Grade II samples. Relative mRNA levels of PDGFR1 were significantly lower in Grade III tumours than in Grade I and Grade II tumour samples.

Our results are similar to literature data concerning the elevated expression values of VEGFR1, VEGFR2, and VEGF. Several studies have found higher values of VEGF in canine mammary gland tumours, and they also suggest the presence of an autocrine loop. Tumour cells are capable of producing VEGF, which can bind to their receptors and trigger their autophosphorylation leading to increased angiogenesis. VEGF is an angiogenic factor, which regulates differentiation, proliferation, and migration of endothelial cells through various pathways, and also has an antiapoptotic function. Restucci et al. (2004) have also found that poorly differentiated, higher-grade tumours have significantly higher levels of VEGFR1 and VEGF than tumours with lower malignancy. The reason is that higher-grade tumours are more aggressive, and can grow faster, which makes them more likely to develop hypoxic or necrotic areas. Therefore, the VEGF-VEGFR autocrine loop is the response of the tumour to the hypoxic stimulus. It may be hypothesized that in the case of malignant tumours, the VEGF-VEGFR axis plays a crucial role in endothelial migration and proliferation, and also in the proliferation of neoplastic cells and tumour growth. In addition, elevated expression of VEGFR and VEGF may increase metastatic potential, partly due to neovascularization and partly because of the increased permeability of the newly formed blood vessels. All in all, increased expression of VEGF and VEGFR are negative prognostic factors that may explain some of the

typical characteristics of high-grade mammary gland tumours, such as rapid growth and early metastasis.

Significant increase could not be observed in VEGFR2 levels in the case of high-grade mammary gland tumours. This suggests that VEGFR2 does not play a significant role in the progression of canine mammary gland tumours (contrary to brain tumours for example), and signalling comes off predominantly via VEGFR1 receptors.

Elevated expression values of PDGF1, EGFR, and ErbB2 are proved in human breast cancer. Elevated expression of PDGFR1/PDGFR-α is associated with increased tumour progression in breast cancer of women. Furthermore, increased expression values of EGFR and ErbB2 have been reported in several types of human breast cancers, leading to larger tumours, lower degree of differentiation, and poorer prognosis. Similarly, elevated levels of mRNA expression of PDGFR1, EGFR, and ErbB2 were observed in our study, mainly in low-grade mammary gland tumour samples. As in humans, these tyrosine kinases increase the malignancy of tumours in dogs, and like in human oncology, they may be potential therapeutic targets in the future. Concerning the elevated expression of ErbB2 (HER-2), our results are consistent with the literature to the effect that increased expression of HER-2 can be found in 29.7% of malignant tumours tested with immunohistochemical methods. One-year, two-years, and overall survival times of the group of dogs with elevated HER-2 expression, treated with surgery alone, were longer than that of the HER-2 negative group, although the difference was not significant in either cases. This fact explains why we could not measure significantly elevated expression levels in the groups with higher-grade tumours.

Our studies confirmed that similarly to other neoplastic diseases, increased expression of MET can also be observed in canine mammary gland tumours, and its levels in Grade III mammary gland tumours are

significantly increased compared to tumours with lower malignancy. These results are consistent with literature data suggesting that increased presence of c-met is associated with a poorer prognosis as it is an oncogenic tyrosine kinase which plays a crucial role in the proliferation, growth, and angiogenesis of tumours, and also affects the metastatic potential.

KIT mutation, which has been reported in several canine tumours, can cause increased proliferation and/or diminished apoptotic potential in tumour cells. Our study confirmed the elevated c-KIT expression in canine mammary gland tumours, which has been reported previously. We also found that levels of c-KIT expression were significantly elevated in Grade III tumours compared to other groups with tumours with lower malignancy, which may partly explain the aggressiveness and poorer prognosis of these tumours.

The presence of c-KIT and c-MET signalling pathways are associated with poorer prognosis and tumour progression in breast cancer of women, confirming the need to develop further anti-c-KIT and anti-c-MET agents. Our results support this in the case of Grade II and Grade III tumours.

Our studies confirmed that oncogenic tyrosine kinases play an important role in the growth, proliferation, and angiogenesis of tumour cells, as well as in the metastatic potential. Our results are the first to show the complex and simultaneous dysregulation of tyrosine kinases in dogs through not only a single receptor, but with the involvement of multiple genes. Our results also confirmed that potential dysregulation of tyrosine kinases should be considered in the treatment of mammary gland tumours, their expression levels should be monitored and targeted inhibition would be beneficial.

The limiting factor of our investigation was the low number of samples, and therefore the low number of mammary gland tumours of different histological types. Nevertheless, our results proved the increased expression and dysregulation of VEGF and several tyrosine kinases (VEGFR1, PDGFR1, c-KIT, and c-MET) in canine mammary gland tumours. Despite the low number of patients, the fact that healthy and cancerous tissue samples were collected from the same patient at the same time makes the results of statistical comparisons much more reliable. Overall, it can be stated that malignant canine mammary gland tumours may be potential targets for studies on tyrosine kinases in the future.

4. New scientific results

1. Our research group was the first in Hungary to investigate Pgp expression and its prognostic value in lymph node samples of dogs suffering from lymphoma. Following the validation of our method, we determined a Pgp expression cut-off value (35%), which has high prognostic and diagnostic values, thus it can be helpful to choose the most effective treatment protocol before starting chemotherapy (individualized medicine).

2. Our research team was the first in Hungary to describe the increased and simultaneous Pgp and p53 expression of canine mammary gland tumours, which partly explains the chemotherapy resistance of these tumours. Most mammary gland tumour samples showed a certain degree of Pgp expression. Our results are consistent with the clinical experience, that canine mammary gland tumours are resistant to Pgp substrate cytostatic agents. This can be explained by the fact that following a potential initial tumour regression, treatment-resistant clones can be

selected and proliferate even in tumours with originally lower levels of Pgp expression, which are no longer sensitive to treatment.

3. We found that p53 immunohistochemistry does not have an appropriate predictive value in the case of canine mammary gland tumours.

4. We also found increased COX-2 expression in advanced and large mammary gland tumours, and detected an unequivocal association between COX-2 expression and tumour size. We demonstrated a trend in the association between elevated expression and poorer clinical outcomes (decreased overall survival time and relapse-free period), as well as the degree of malignancy. We also confirmed the prognostic value of COX-2 immunohistochemistry in canine mammary gland tumours, and determined a cut-off value of 50%, which turned out to have a pronounced prognostic value. Based on our results, canine mammary gland tumours may be potential targets for COX-2 inhibitor therapy.

5. Our study confirmed the overexpression and dysregulation of several oncogenic tyrosine kinases (VEGFR1, PDGFR1, c-KIT, and c-MET) and VEGF in canine mammary gland tumours. Our research team was the first to demonstrate complex dysregulation of the listed oncogenes in the case of canine mammary gland tumours. Based on our results, canine mammary gland tumours may be potential targets for tyrosine kinase inhibitor therapy.

5. Publications based on the results of the doctoral study

5.1. Publications in peer-reviewed scientific journals with impact factors

Koltai Zs., Vajdovich P.: **Expression of multidrug resistance membrane transporter (Pgp) and p53 protein in canine mammary tumours.** Acta Vet Hung. 62(2). 194-204, 2014. IF (2014): 0.646

Koltai Zs., Szabó B., Jakus J., Vajdovich P.: **Tyrosine kinase expression analyses in canine mammary gland tumours - A pilot study.** Acta Vet Hung. 66(2). 294-308, 2018. IF (2018): 1.059

Vajdovich P., Koltai Zs., Dékay V., Kungl K., Harnos A.: **Evaluation of Pgp (MDR1) immunohistochemistry in canine lymphoma - prognostic and clinical aspects.** Acta Vet Hung. 66(2). 309-328, 2018. IF (2018): 1.059

Koltai Zs., Vajdovich P., Dékay V.: **A ciklooxigenáz-2 (COX-2) enzim expressziójának jelentősége a kisállatok daganatos kórképei esetén**. MÁL. 136(10). 579-587, 2014. IF (2014): 0.185

Koltai Zs., Vajdovich P., Jakab Cs., Szabó B.: **A tirozinkináz-gátlás jelentősége a kisállatok daganatos kórképei esetén.** MÁL. 139(8). 465-472, 2017. IF (2017): 0.196

5.2. Publications in peer-reviewed scientific journals

Dékay V., Vajdovich P., Koltai Zs.: **A COX-2 enzim expressziójának immunhisztokémiai módszerrel történő vizsgálata kutyák emlődaganataiban.** Kisállatpraxis. 13(5). 200-206, 2012.

5.3. Conference presentations

Koltai Zs., Dékay V., Vajdovich P.: **A COX-2 expressziójának vizsgálata kutyák emlődaganataiban.** MTA Állatorvostudományi Bizottsága, Akadémiai beszámoló, 2011.

Szabó B., Koltai Zs., Tóth B., Vajdovich P.: **Emlődaganatos és lymphomás kutyák betegségének lefolyását meghatározó fehérjék expressziójának vizsgálata.** MTA Állatorvostudományi Bizottsága, Akadémiai beszámoló, 2011.

Koltai Zs.: **A COX-2 és antagonistái szerepe az állatorvosi onkológiában.** Magyar Állatorvosi Onkológiai Társaság VII. konferenciája, 2012.

Szabó B., Machut J., Koltai Zs., Vajdovich P.: **Receptor tirozin-kinázok mRNS-expressziójának változása kutyák emlődaganataiban**. MTA Állatorvostudományi Bizottsága, Akadémiai beszámoló, 2012.

Szabó B., Koltai Zs., Vajdovich P.: **Expression of receptor tyrosine kinases in canine mamary gland tumours.** ESVONC Annual Congress 2013.

Dékay V., Karai E., Verebélyi T., Koltai Zs., Vajdovich P**.: A multidrogrezisztenciafehérje (MDR1) immunhisztokémiai és funkcionális vizsgálatának összehasonlítása lymphomás, emlődaganatos és mastocytomás kutyák vizsgálata során.** MTA Állatorvostudományi Bizottsága, Akadémiai beszámoló, 2015.

6. Publications not closely related to the topic of doctoral research

6.1. Publications in peer-reviewed scientific journals with impact factors

Lehner L., Czeibert K., Koltai Zs., Jakab Cs.: **Frontalis meningeoma eltávolítása bilateralis transzfrontalis feltárással kutyában.** MÁL. 141(9). 533-545, 2019., IF (2018/2019): 0.143

6.2. Conference presentations

Szécsényi D., Vajdovich P., Koltai Zs.: **A terápiarezisztencia vizsgálata kutyák kemoterápiás kezelése során.** MTA Állatorvostudományi Bizottsága, Akadémiai beszámoló, 2006.

Vajdovich P., Besze A., Perge E., Koltai Zs.: **Lymphomás kutyák csontvelővizsgálati eredményeinek összefügései a betegek klinikai és kórszövettani leleteivel.** MTA Állatorvostudományi Bizottsága, Akadémiai beszámoló, 2011.

Vajdovich P., Koltai Zs., Szendi E: **A doxorubicin toxikus hatásának mérséklési lehetősége retrospektív vizsgálatok alapján. Dóziscsökkentés vagy előkezelés?** MTA Állatorvostudományi Bizottsága, Akadémiai beszámoló, 2012.

Tóth B., Koltai Zs., Szendi E., Vajdovich P.: **Tapasztalatok a SYSMEX XT-2000 IV haematológiai automatával a klinikumban.** MTA Állatorvostudományi Bizottsága, Akadémiai beszámoló, 2015.