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Prospective Study of Tumor Markers as Prognostic Factors in the Histopathological Differential Diagnosis of Mammary Gland Neoplasms in Female Canines

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1. Introduction

Cancer constitutes a major problem in animal pathology and is a subject of intensive research. The aim of this study was to gain comprehensive knowledge of cancer biology. Some animal tumors are also considered good research models in comparative oncology. Literature and own studies show that dogs often suffer from skin cancer and mammary gland tumors (Moulton, 1990). The incidence of mammary gland tumors in female canines is second only to skin cancer (Misdorp & Meuten, 2002; Ramalho et al, 2006). They most commonly appear between 6 and 10 years of age (Misdorp & Meuten, 2002; Hampe & Misdorp, 1974; Kubiak, 200). There are also documented cases of such tumors as early as at the age of two years and, very rarely, in males (Moulton, 1990). Histopathological examination in about 40-50% of cases shows malignant tumors, most of which are of epithelial origin (Rutteman, 2001). These cancers originate from epithelial follicles or ducts and take forms of papillary adenocarcinomas, simple or complex tubular and solid cancers (Misdorp & Meuten, 2002). There are also tumors derived from myoepithelial cells and mesenchymal tissue. In veterinary medicine there is a constant search for prognostic factors and predictors that allow for proper evaluation of the disease state, survival, susceptibility to treatment and risk of relapse. Recognized factors are: age of the animal, sentinel lymph node status, histological type of tumor and a histological grade of differentiation of malignancy. Beside the fundamental method in cancer diagnosis, which is the histopathological examination, additional information useful in this regard is provided by research on cancer biology, including, among others, the study of tumor markers. In histological examination of a tumor, markers of proliferative activity such as mitotic index, thymidine labeling index, percentage of cells in the S phase of cell cycle, expression of nuclear antigen Ki-67 and proliferating cells nuclear antigen (PCNA) are also taken into account. The following factors are also examined: expression of estrogen receptors, growth factor receptors such as: epidermal growth factor receptor (EGFR), insulin growth factor receptor (IGFR), growth hormone (GH),markers of high risk of metastasis: plasminogen activator and cathepsin D, oncogenes and tumor suppressor genes: c -erbB-2, c-myc, p53,

BRCA-1, BRCA-2, markers of tumor angiogenesis, and heat shock proteins (Koda et al, 2004; Niwińska, 1995; Olszewski, 1994). Diagnosis of neoplasms of the mammary gland in canines does not usually pose a problem, but in some cases there may be difficulties. Targeted diagnostic immunohistochemistry using a panel of antibodies makes it easier to determine the correct diagnosis. Difficulties encountered during routine diagnosis of neoplasms of the mammary gland in female dogs relate to assessing the origin of the tumor and its biology. Immunophenotyping can be helpful in the diagnostic process and tumor markers (expression of nuclear antigen Ki-67 and expression of estrogen receptors and the p53 protein) carry useful information, but determination of malignancy is still based on morphological criteria, such as mitotic index, which together with the histopathological criteria are the most important factors differentiating benign tumors from those of malignant nature;. In veterinary oncology there is a constant search for new tumor markers that could aid in differential diagnosis of mammary gland neoplasms in female canines. The aim of research on the mammary gland neoplasms in female dogs is to extend the panel of tumor markers by addition of new markers such as COX-2, P-gp, Hsp90 and Hsp70 and to incorporate them into routine diagnostics. Occurrence of mammary tumors in females as well as potential to use canine models in comparative pathology of these tumors makes it an interesting research material. It is known that the degree of malignancy is positively correlated with proliferative activity. Relationship between the expression of estrogen receptors and the degree of malignancy is not entirely clear, although most authors believe that expression of these receptors is greater in benign tumors. COX-2 is expressed in many canine tumors such as canine kidney, bladder, prostate, and mammary gland cancers, but there is no data on the relationship between expression of COX-2 and other markers, even though such relation would seem logical because the expression of COX-2 may be related to prognosis by indirect immunosuppressive effect and inhibition of apoptosis. Literature on the expression of heat shock protein or glycoprotein - P in breast cancer is very scarce. There is evidence of Hsp27, Hsp70 and Hsp90, as well as glycoprotein – P expression, which may influence susceptibility to therapy. One would expect a correlation between proliferative activity, degree of malignancy and expression of estrogen receptors, but no data is available on such relationship. The aim of the study was to analyze the role of classical and new tumor markers in the histopathological differential diagnosis of mammary gland neoplasms in female dogs. The objective of the present study was to investigate the expression of cyclooxygenase - 2 and heat shock proteins in canine mammary gland tumors and their correlations with histological type of tumor, degree of its histological malignancy, proliferative activity, estrogen receptor expression, infiltration by cells, as well as P-gp and p53 protein expression.

2. Materials, methods and results

2.1 Materials

Material for the investigation comprised 133 tumor samples of the mammary gland collected from female canines during surgical procedures performed at Warsaw Veterinary Clinics and Small Animal Clinic of the Department of Clinical Sciences, Faculty of Vetrinary Medicine, Warsaw University of Life Sciences – SGGW. Tumor samples were fixed in 8% formalin buffered with phosphates. After 24h fixing, material was dehydrated in a series of increasing concentrations of alcohol and embedded in paraffin. Paraffin blocks were cut into serial sections of 4μm in thickness.

2.2 Methods

Then, they were stained using proper methods. In sections stained with the routine H&E method, the following determinations were carried out: type of neoplasm (Misdorp et al, 1999), tumor grade including tubule formation, intensity of division and the degree of neoplastic cell differentiation (Misdorp & Meuten, 2002), mitotic index defined as a mean number of mitoses in neoplastic cells counted in 10 fields of vision at the lens magnification of 400x (surface field 0.17mm^2). Paraffin sections on slides covered with 2% saline solution in acetone, at the temperature of 42^{0}C were used for immunohistochemical methods. In immunohistochemical reactions, the following antibodies, properly diluted in 1% BSA (Sigma), were used: mouse monoclonal antibody against human nuclear antigen Ki-67 (Dako) diluted 1:75 (Nieto et al, 2000), mouse monoclonal antibodies against p53 (Dako) human protein, diluted at a ratio 1:25 (Gamblin et al, 1994; Rodo, 2007), mouse monoclonal antibodies against alpha (Dako) human estrogen receptor, diluted at a ratio 1:35 (Mulas et al, 2005), mouse monoclonal antibodies against COX-2 (Dako) human receptor, diluted at a ratio 1:100 (Queiroga et al, 2007; Doré et al, 2003; Soslow et al, 2000), mouse monoclonal antibodies against Hsp70 (Novocastra) human heat shock proteins, diluted at a ratio 1:40 (Romanucci et al, 2006), mouse monoclonal antibodies against Hsp90 (Novocastra) human heat shock proteins, diluted at a ratio 1:40 (Romanucci et al, 2006) and mouse monoclonal antibodies against P-glycoprotein (Sigma), diluted at a ratio 1:100 (Petterino et al, 2006). Sections were deparafinized in xylene and rehydrated in increasing concentrations of alcohol. Then, they were placed in a buffer with a pH of 6 (Dako). In order to uncover the antigen epitope, sections were warmed up in a microwave oven (1x5 min at 600 W, 2x5 min at 300 W). Then, they were cooled for 20 min. After two washings in distilled water (5 min), slides were washed in TRIS-buffered saline with pH of 8 (Sigma) and incubated with the primary antibody for 1h at room temperature. Then, the preparations were washed with TRIS buffer for 10 min. The En Vision + ™ System (Dako) was used for visualization. After 30 min of incubation with reagent, the slides were washed with TRIS buffer and a solution of diaminobenzidine (DAB, Dako), prepared according to the procedure supplied by the producer, was put on slides. The degree of slide staining was controlled. They were washed in tap water and stained with Ehrlich hematoxylin for 5 min, contrasted in 1% acid alcohol and washed again in tap water. Then, slides were dehydrated in graded alcohol concentrations, passed through xylene and fixed in DPX mounting medium (Gurr®). Computer image analysis and Lucia v. 4.21 software were used for interpretation of the results of Ki-67, ER, p53, COX-2, Hsp70, Hsp90 expression; using those facilities we could count the number of neoplastic cells featuring stained cytoplasm per 1,000 neoplastic cells. Results were analyzed using the SPSS 12.0 program. To determine whether differences for a few independent traits were significant, Kruskal-Wallis test was used. This test is an equivalent to the test of variance for traits without normal distribution. Two-sided correlations were performed using Spearman correlation test. The differences were deemed statistically significant at $P \leq 0.05$.

2.3 Results

Investigated material contained 14 adenomas (Fig.1), 66 complex carcinomas (adenocarcinomas), 47 simple carcinomas (adenocarcinomas) (Fig. 2, Fig. 3) and 6 solid carcinomas. The number of cancers with a defined grade amounted to, respectively, 1st grade – 48, 2nd grade – 39 and 3rd grade – 32. Mammary gland neoplasms were excised from female dogs belonging to 18 breeds in the ages between 3 and 16 years.

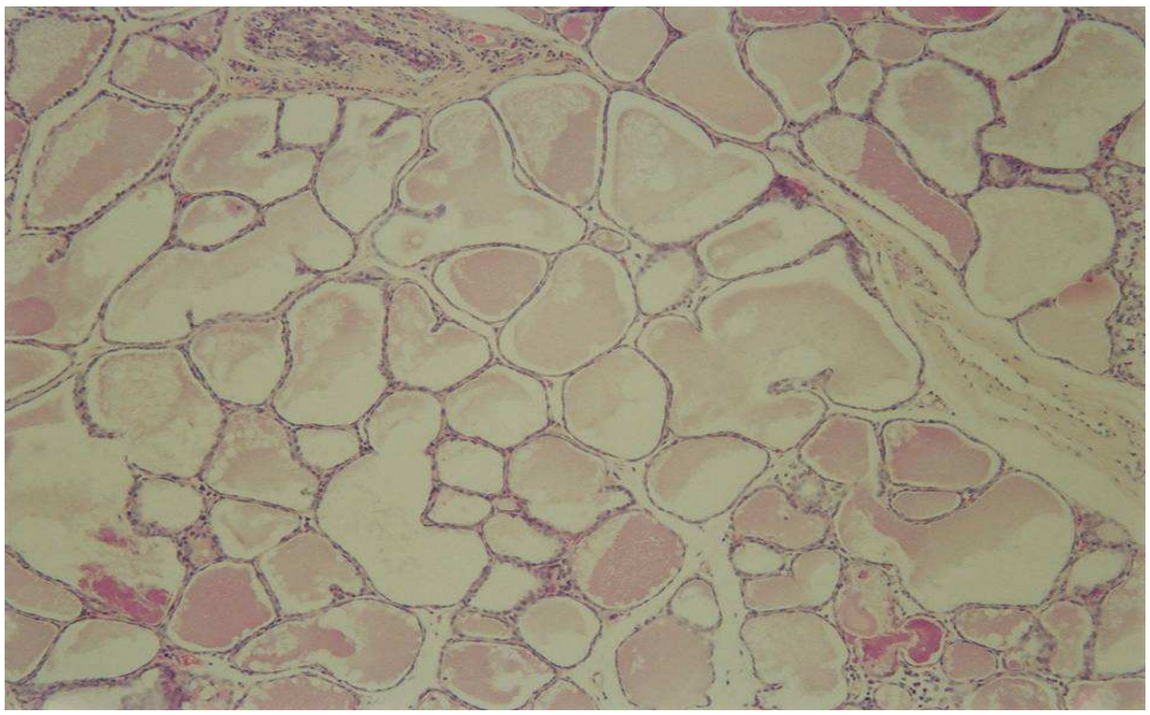


Fig. 1. Adenoma, H&E method x 20

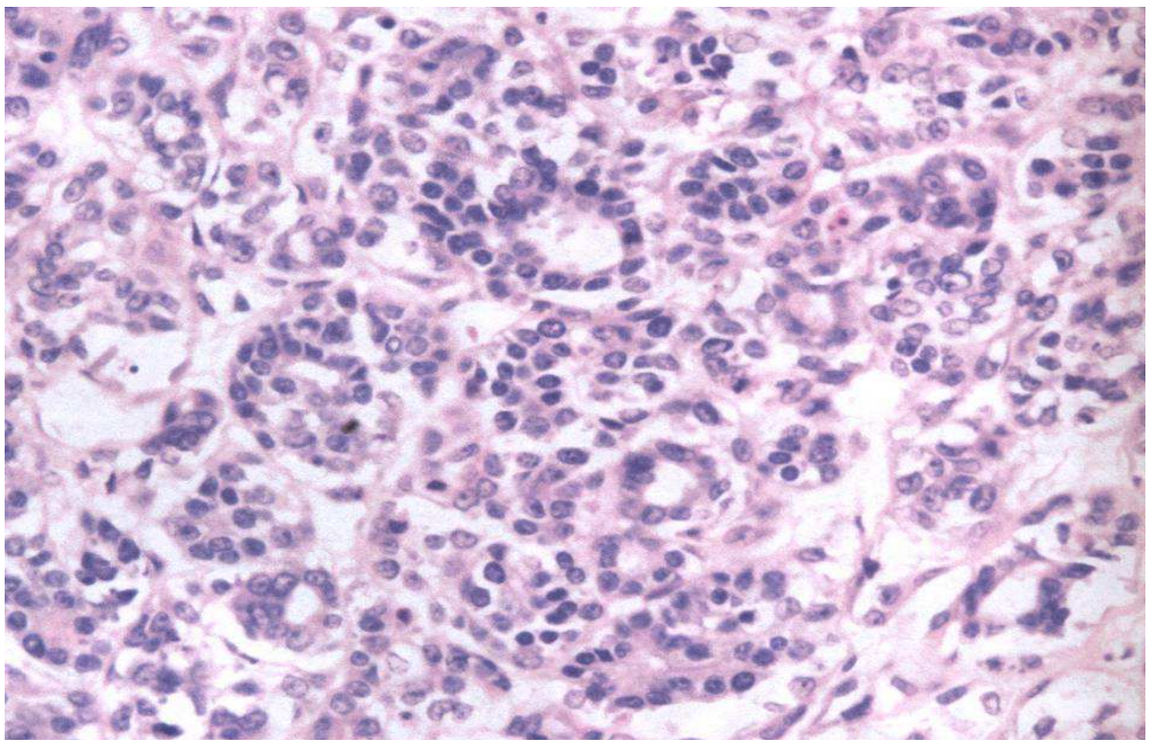


Fig. 2. Adenocarcinoma simplex, H&E method x 20

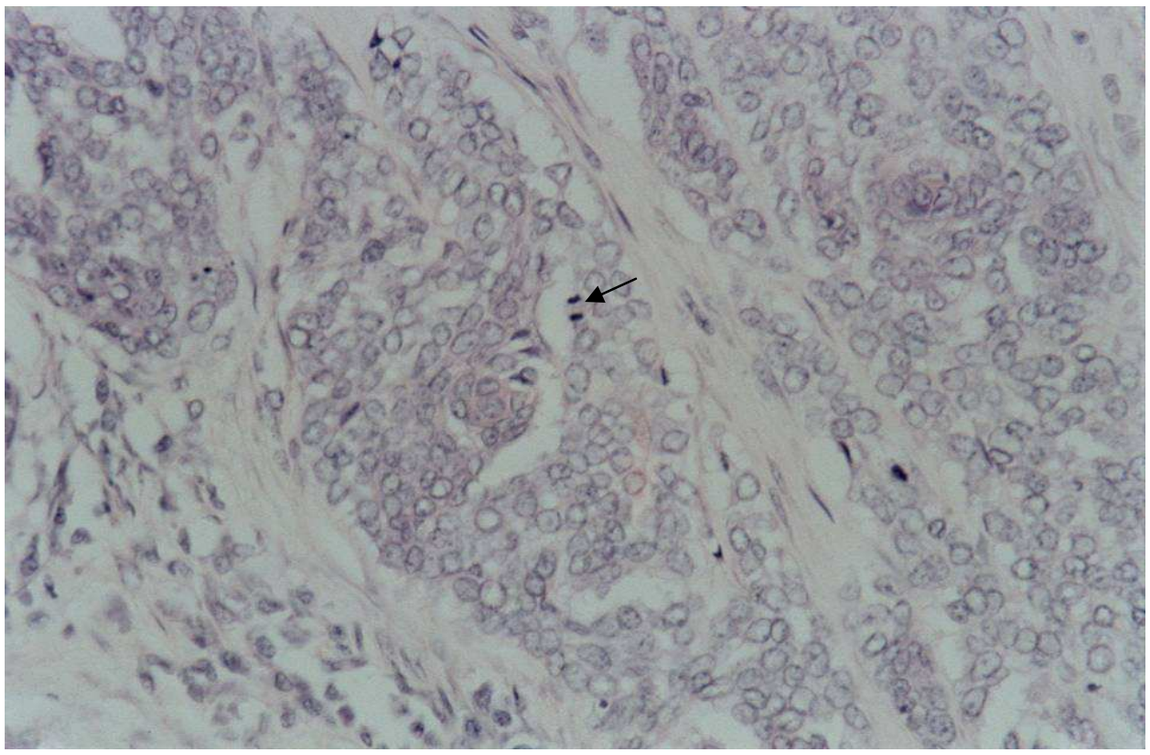


Fig. 3. Adenocarcinoma simplex, H&E (40x). The following mature tissues are visible: pathologic mitotic figures

2.3.1 Relationship between age of bitches and the grade of malignancy and histological type of tumor of epithelial origin

Dogs were divided into three age groups: <8 years, 8 -12 years and >12 years. In the group of bitches below the age of 8, majority (61.1%) consisted of tumors with the lowest histological grade of malignancy (1st). In the oldest group, 1st and 2nd grade tumors in the 1st and 2nd accounted for 77.8%. In the entire pool of studied tumors in all age groups, the largest share consisted of tumors with the lowest degree of histological malignancy (40.4%) (Table 1). Assessment of the contribution of individual types of tumors at different ages showed that in bitches younger than 8 years the most common findings were adenomas (21.7%) and complex carcinomas (56.5%) and in those over 12 years simple carcinomas occurred most often (55.0%) (Table 2).

Age of bitches	Tumor grade			Total
	Io	IIo	IIIo	
<8 lat (n=18)	11 (61,1%)	3 (16,7%)	4 (22,2%)	18
8-12 lat (n=83)	30 (36,0%)	29 (35,0%)	24 (29,0%)	83
>12 (n=18)	7 (38,9%)	7 (38,9%)	4 (22,2%)	18
total (n=119)	48 (40,4%)	39 (32,8%)	32 (26,8%)	119

Table 1. Incidence of malignancies of various grades in bitches in different age groups

Age of bitches	Types of tumors				Total
	Adenoma	Carcinoma solidum	Adenocarcinoma simplex	Adenocarcinoma complex	
<8 lat	5 (21,7%)	1 (4,3%)	4 (17,4%)	13 (56,5%)	23
8-12 lat	7 (7,8%)	4 (4,4%)	32 (35,6%)	47 (52,2%)	90
>12 lat	2 (10,0%)	1 (5,0%)	11 (55,0%)	6 (30,0%)	20
Total	14 (10,5%)	6 (4,5%)	47 (35,3%)	66 (49,6%)	133

Table 2. Occurrence of individual types of epithelial neoplasms in different age groups in bitches

2.3.2 Results of proliferative activity

The value of mitotic index differed significantly between types of tumors. The lowest proliferative activity was observed in adenomas, the highest in simple and solid carcinomas. The highest proliferative activity was found in tumors in the 3rd grade of malignancy, the lowest in the tumors in the 1st grade (Fig.4). Expression of Ki-67 protein was observed in the nuclei of neoplastic cells that have undergone division (Fig.5). Statistical analysis shows significant differences between particular types of tumors. The lowest number of cells exhibiting Ki-67 protein expression was observed in adenomas, the highest in the solid and simple carcinomas and in tumors with the highest histological grade of malignancy (3rd).

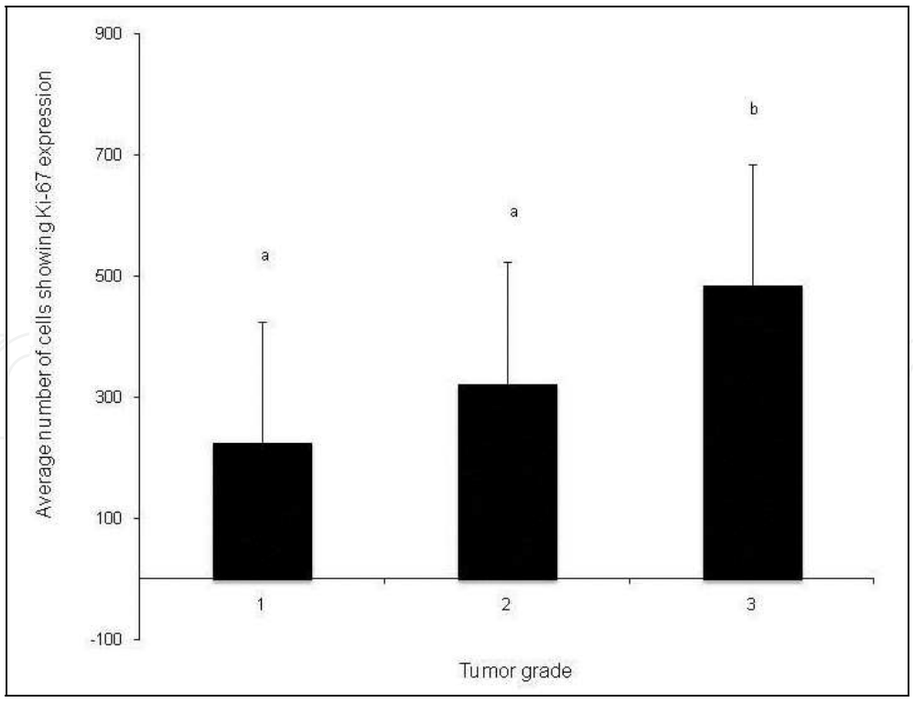


Fig. 4. Average number of cells showing Ki-67 expression depending on the tumor grade. Letters (a, b, c) above the columns show that the differences between mean values were statistically significant (P≤0.05).

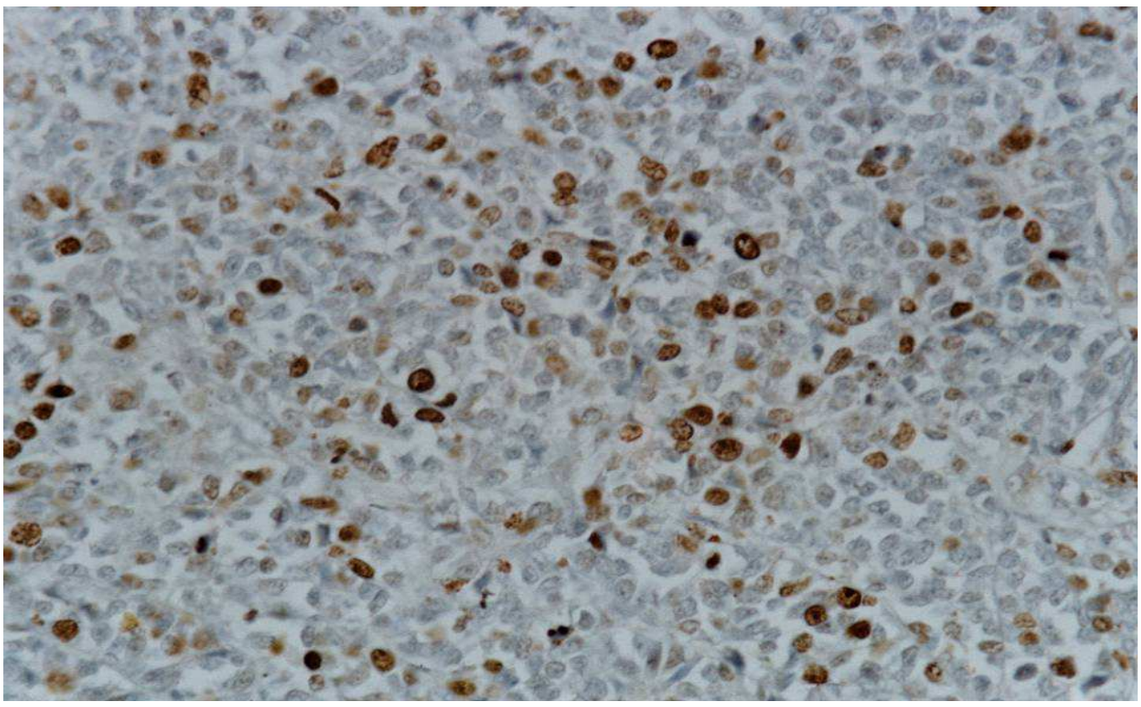


Fig. 5. Adenocarcinoma simplex, expression of Ki-67, immunohistochemical method. (40x)

2.3.3 Results of cellular infiltration in tumors of epithelial origin

In most cases, infiltrates were located predominantly in the stroma at the periphery of the tumor, and, in rare cases, occurred in the glandular alveoli. Inflammatory infiltrates consisted of lymphocytes, neutrophils and macrophages. The intensity of cellular infiltration was assessed on a four-level scale:: -/+; +; ++; +++. Statistical analysis indicates no correlation between the age of the dog and the intensity of cellular infiltration in the tumor. It is worth noting that, in bitches between the ages of 8 to 12 years, most tumors exhibited the intensity of infiltration at the first (+) level , which constituted 50% of tumors in this age group. A significant relationship was found between the intensity of cellular infiltration and the type of tumor. In 50% of solid cancers, there was no cellular infiltration. Only 33% of these tumors exhibited the presence of cellular infiltration at the first level (+), and 16.7% on the second level (++). Among the complex cancers, the largest group consisted of those, in which cellular infiltration was found at the first (+) and the second (++) level. The highest average intensity of cellular infiltration was found in complex carcinomas, followed by simple carcinomas, adenomas and solid carcinomas. Specific differences were seen between adenomas and complex carcinomas (P=0.007) and between solid carcinomas and complex carcinomas (P=0.005). While examining the relationship between intensity of cellular infiltration and histological grade of malignancy, no significant differences were revealed. But it may be noted that among tumors of all histological grades of malignancy, the largest group consisted of cancers with the first level of cellular infiltration. While analyzing the average intensity of cellular infiltration in tumors with various histological grades of malignancy no significant differences were found between groups with the exception of the tumors in the 3rd grade, where a significant difference was observed (P=0.023).

2.3.4 Results of p53 expression in neoplasms of mammary gland

Expression of p53 protein was observed in the nuclei of cancer cells (Fig.6). Positive reaction of p53 protein was observed in 70 (52.6%) of all tumors. Expression of p53 protein was found in 4 adenomas. The largest group of tumors positive for p53 protein were complex and simple cancers. Solid cancers belonged to a group, which rarely exhibited a positive reaction for nuclear p53. Analysis of the average number of cells with a positive reaction for p53 showed its highest expression in the case of complex cancers, as well as in 1st and 2nd grade tumors (Fig.7). The lowest level of expression of p53 protein was found in adenomas.

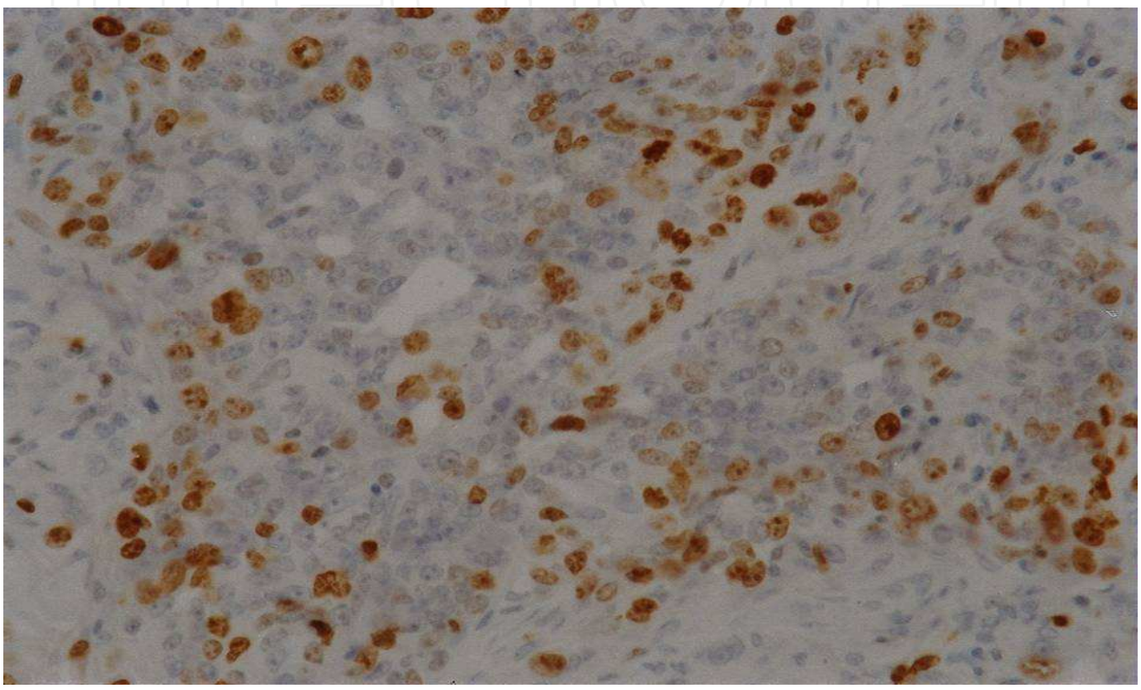


Fig. 6. Adenocarcinoma simplex, expression of p53, immunohistochemical method. (40x)

No statistically significant differences were shown between the examined groups. Comparing the ages of bitches, the highest average number of cells expressing p53 protein was found in tumors excised from younger dogs. However, there were no statistically significant differences among the age groups.

2.3.5 Results of estrogen receptor expression in neoplasms of mammary gland

Estrogen receptor expression was detected in the nuclei of tumor cells, but it was also seen in the cytoplasm. Cytoplasmic reaction was considered to be nonspecific. Among all tumors of epithelial origin, expression of estrogen receptors was found in 54 (40.6%) , and no reaction was noted in 79 (59.4%). Expression of estrogen receptors was most commonly found in complex cancers (43.9%) (Fig.8) , followed by simple cancers (42.6%) (Fig.9, Fig.10) and adenomas (28.6%), while solid cancers rarely expressed them (16.7%). The highest expression of estrogen receptors was found in simple carcinomas as well as in 3rd grade tumors, but no statistically significant differences were found between study groups (Fig. 11). Analysis of the average number of cells showing positive expression of estrogen

receptors reveals that the level of expression increases with the histological grade of malignancy. It was also found that most tumors expressing estrogen receptors came from dogs younger than 8 years. A positive correlation was found between mitotic index and expression of estrogen receptors in specific types of cancer and statistically significant differences between tumor characteristics were demonstrated (P=0.042).

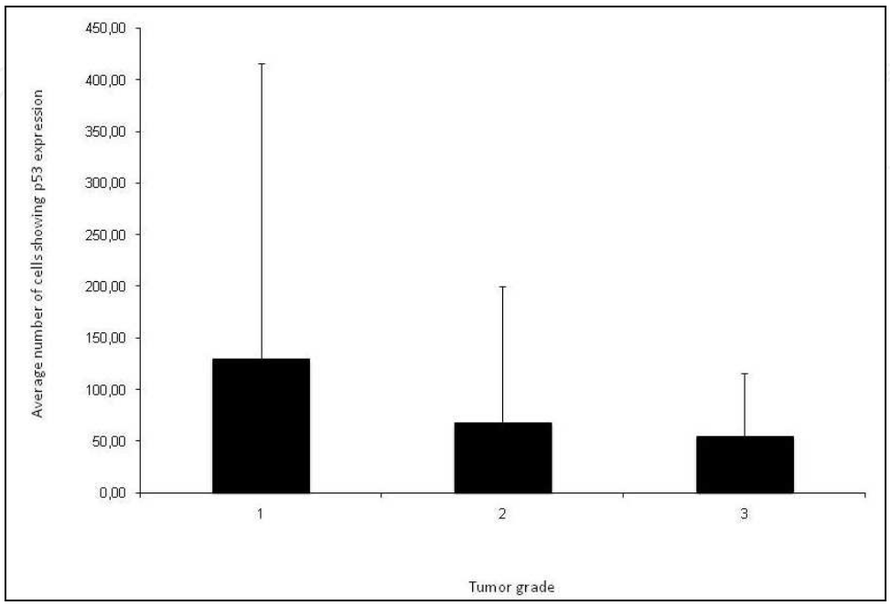


Fig. 7. Average number of cells showing p53 expression depending on the tumor grade.

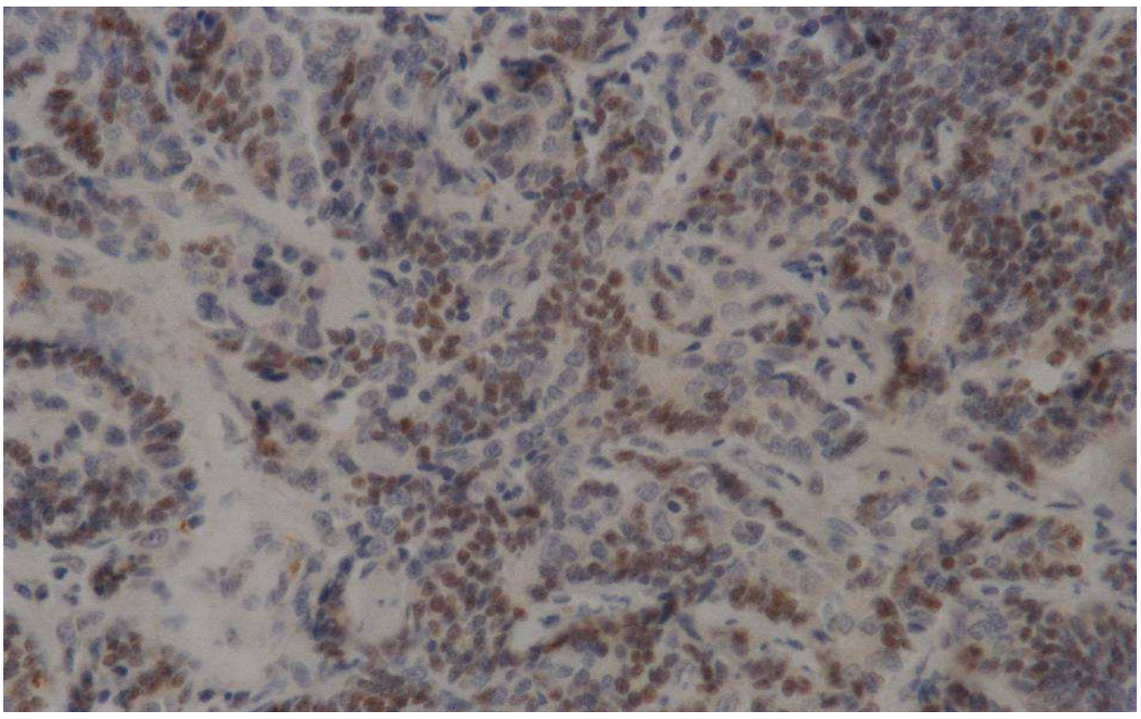


Fig. 8. Adenocarcinoma complex, expression of ER, immunohistochemical method. (40x)

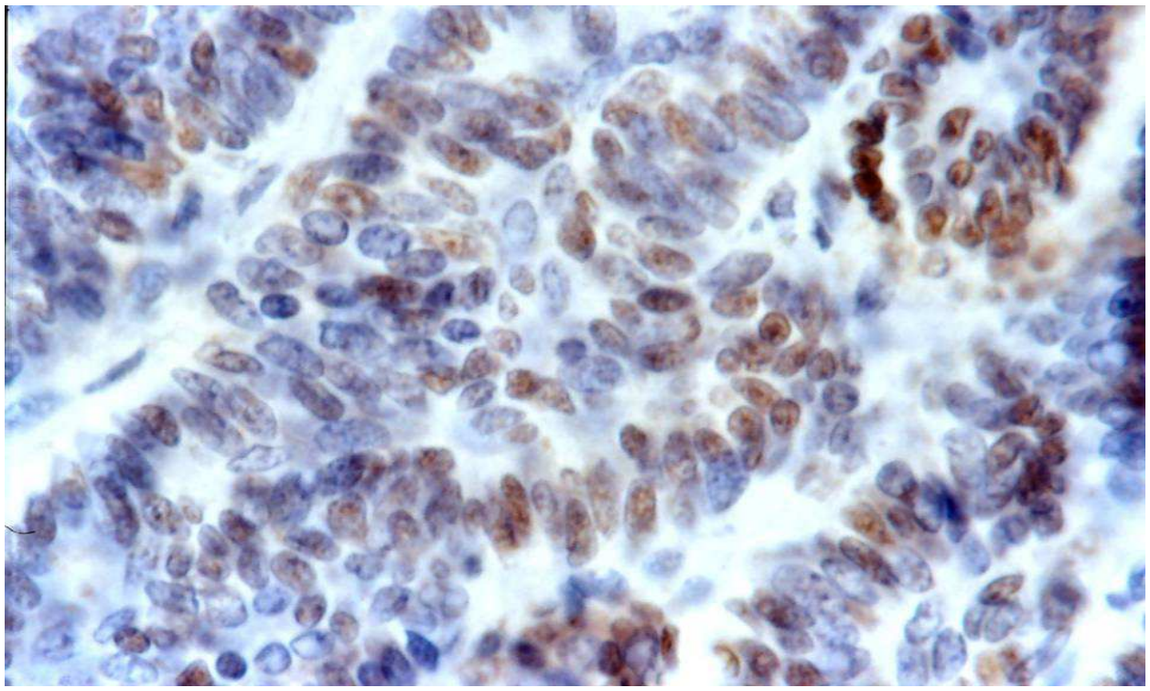


Fig. 9. Adenocarcinoma simplex, expression of ER, immunohistochemical method. (100x)

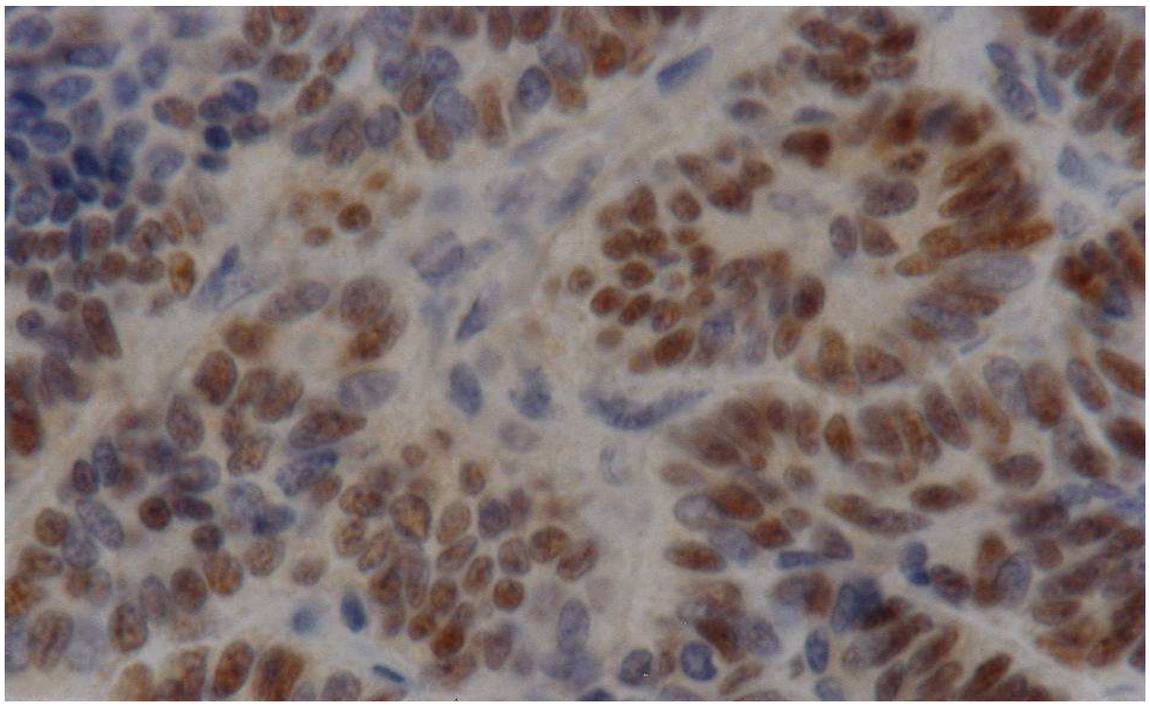


Fig. 10. Adenocarcinoma simplex, expression of ER, immunohistochemical method. (100x)

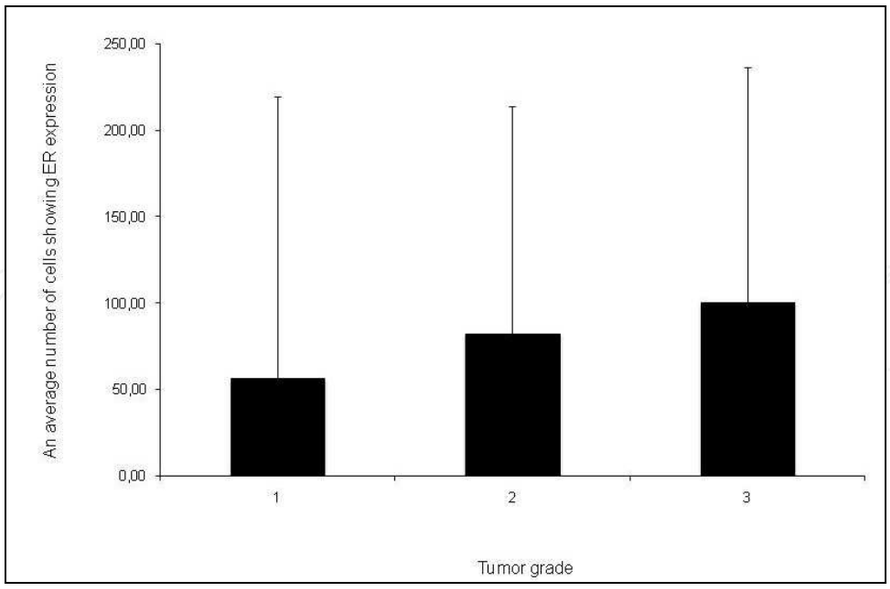


Fig. 11. An average number of cells showing ER expression depending on the tumor grade

2.3.6 Results of cyclooxygenase-2 expression in neoplasms of mammary gland

Expression of cyclooxygenase – 2 was observed in the neoplastic cell cytoplasm. It was confirmed in 13 of 14 adenomas. Similar results were obtained in solid cancers (5 of 6) (Fig. 12). Positive COX–2 reaction was found in 36.9% of simple carcinomas (Fig.13). Of all tumors, which exhibited a positive response (122), complex tumors demonstrated the highest percentage (48.4%). Statistical analysis, however, failed to demonstrate any significant differences regarding the COX–2 expression between the individual types of carcinoma (P = 0.978). According to the analysis of histological malignancy grades, the highest expression was found in 3rd grade cancers and statistical significance was confirmed for the individual histological malignancy grades (P = 0.047) (Fig. 14, Fig. 15). Furthermore, higher COX-2 expression level was demonstrated in carcinomas with higher mitotic index; statistical analysis demonstrated highly significant differences between neoplasms with high versus low mitotic index (P = 0.009) (Fig.16). The investigation into the relationship between expression of Ki-67 nuclear antigen and the COX–2 expression level did not reveal statistical significance (P = 0.614) (Fig. 17). Analysis of the relationship between the p53 protein expression and COX–2 expression level demonstrated a statistically significant correlation between these features (P = 0.034) (Fig. 18). An average number of cells expressing p53 protein was higher in carcinomas with higher COX–2 expression. In addition, experiments explored the relationship between Hsp70 protein and cyclooxygenase – 2 expression levels. The study proved a statistically high correlation between the investigated neoplastic characteristics (P = 0.006). The lowest Hsp70 protein expression was found in carcinomas exhibiting second degree COX–2 expression (Fig. 19). Analysis of the relationship between representative neoplastic features demonstrated statistically significant, positive correlations between COX–2 expression, mitotic index (P = 0.009) and p53 protein expression (P = 0.003); on the other hand, no correlation was found with regard to the Ki-67 nuclear antigen expression (P = 0.686). There was no correlation between the estrogen receptor expression and the above mentioned parameters. The highest expression of estrogen receptors was found in simple cancer types.

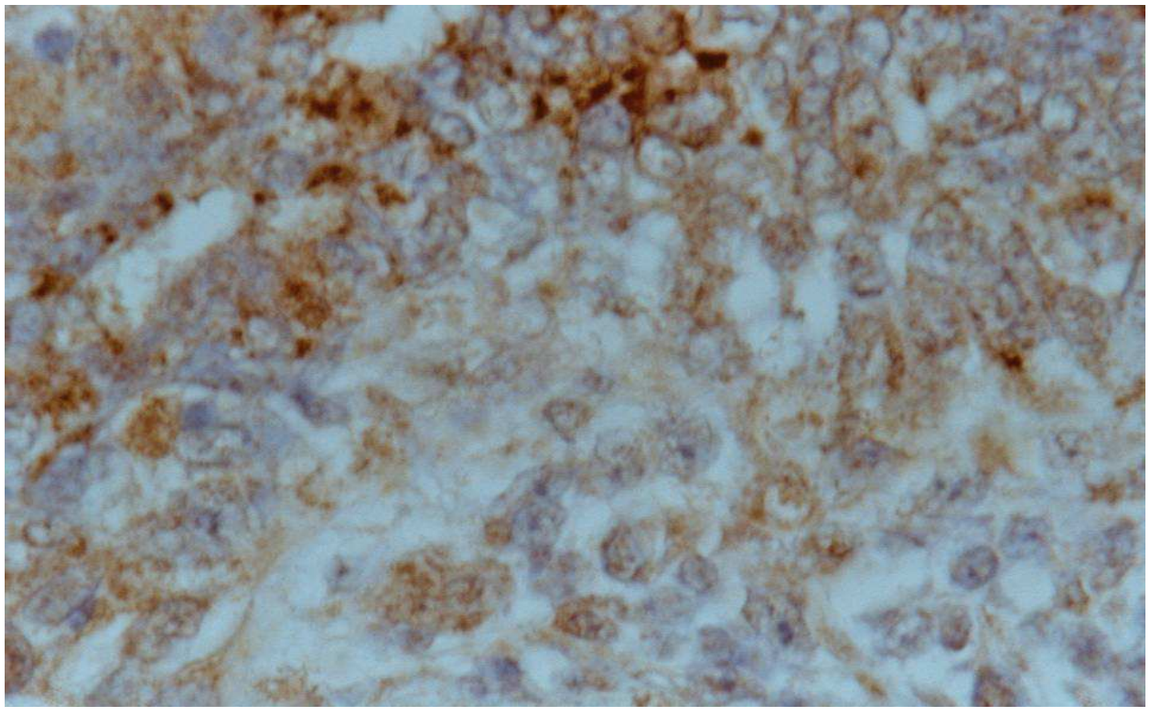


Fig. 12. Carcinoma solidum, expression of COX–2, immunohistochemical method. (100x)

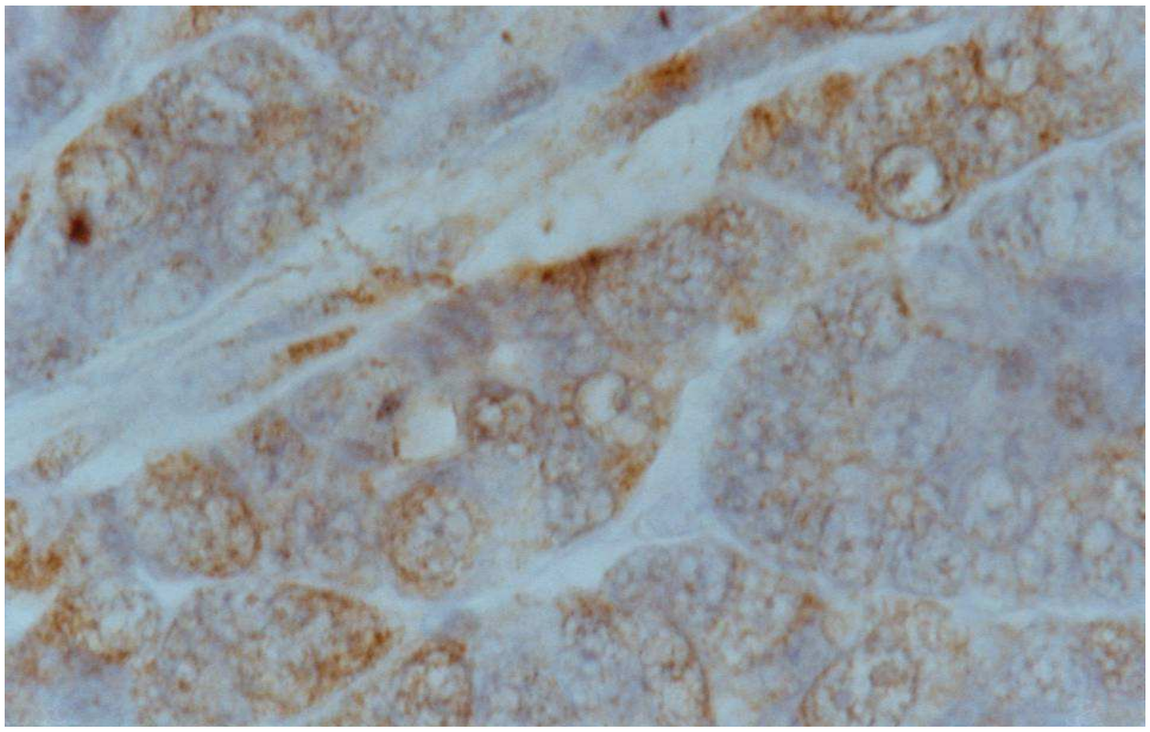


Fig. 13. Adenocarcinoma simplex, expression of COX–2, immunohistochemical method. (100x)

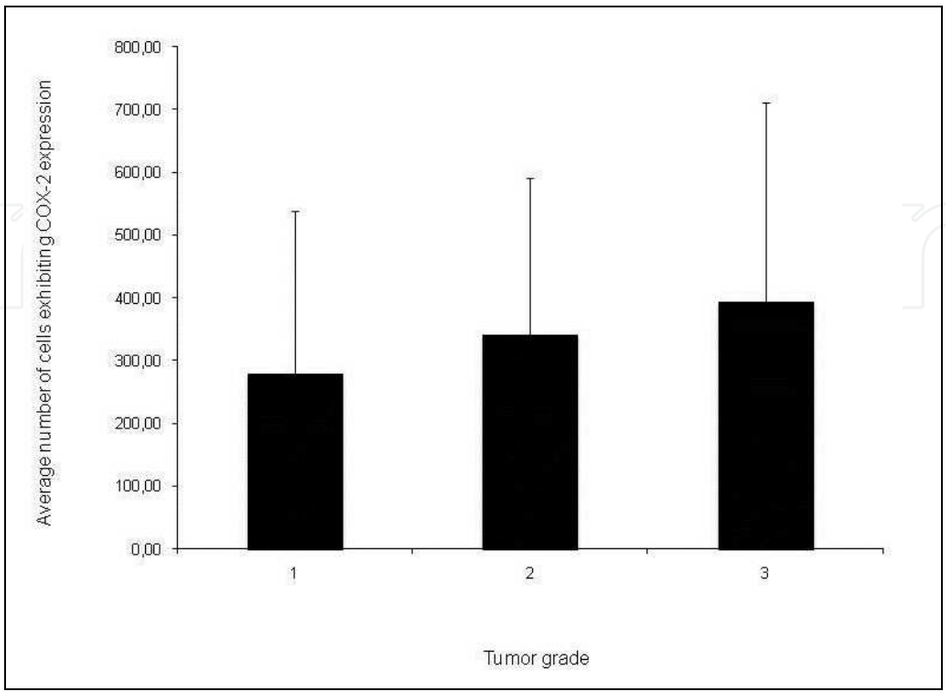


Fig. 14. Average number of cells exhibiting COX-2 expression depending on the tumor grade.

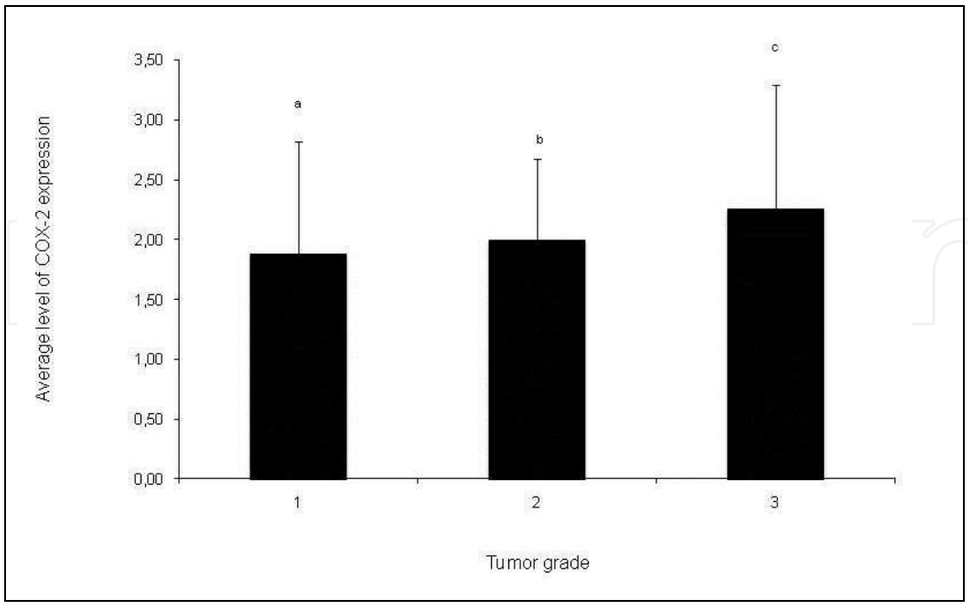


Fig. 15. Average level of COX-2 expression depending on tumor grade. Letters (a, b, c) above the columns show that the difference between means was statistically significant (P≤0,05).

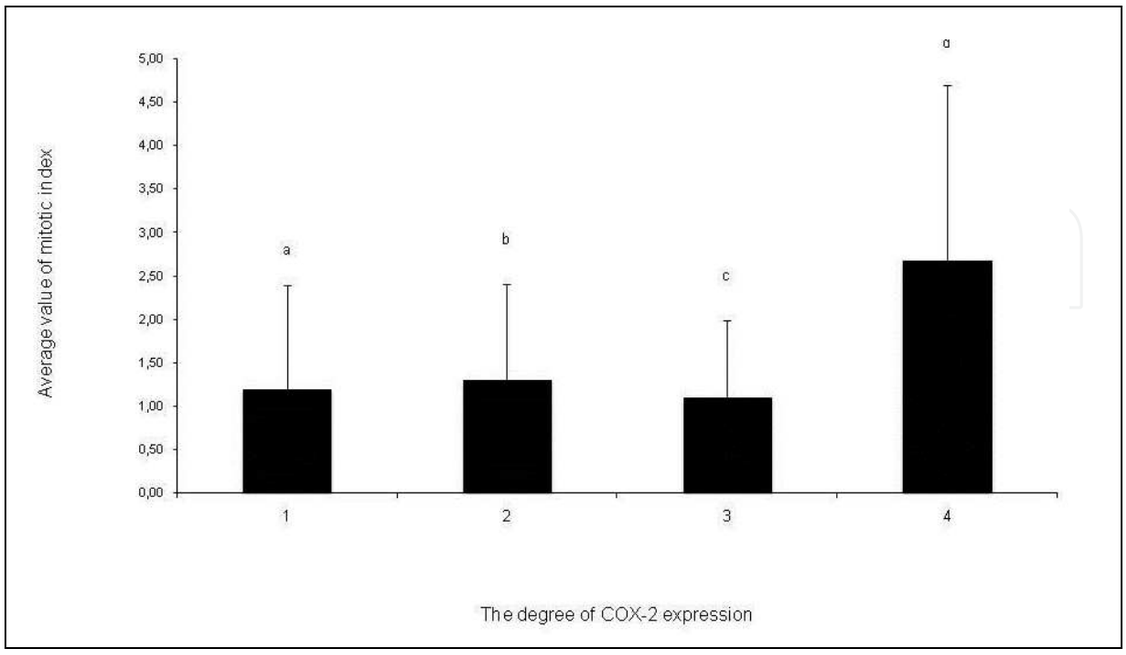


Fig. 16. Average value of mitotic index in tumors with different degrees of expression of cyclooxygenase – 2. Letters (a, b, c, d) above the columns show that the difference between means was statistically significant (P≤0.05).

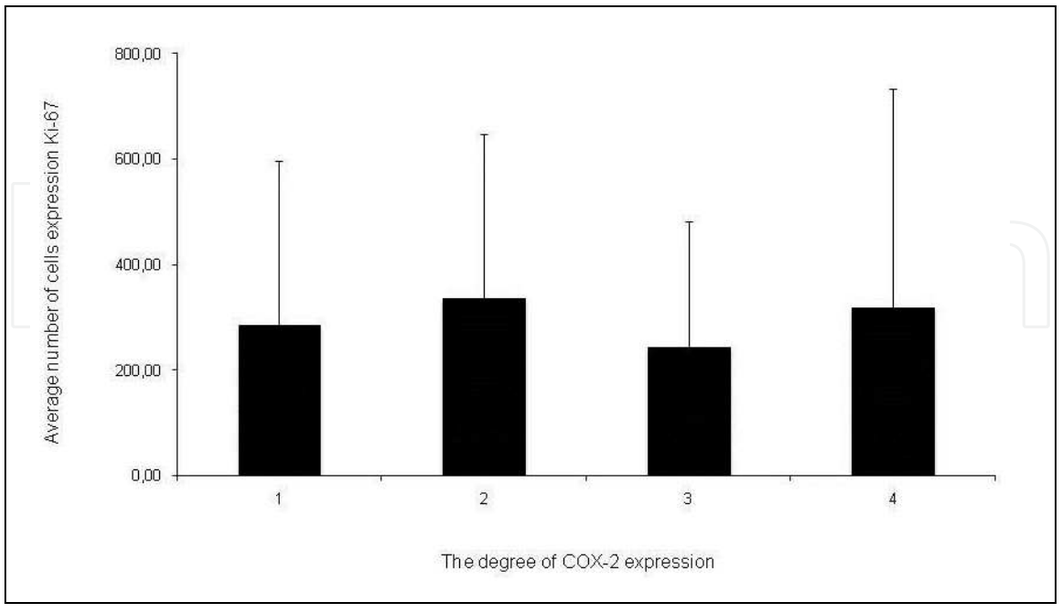


Fig. 17. Average number of cells expressing Ki-67, depending on the degree of expression of cyclooxygenase – 2.

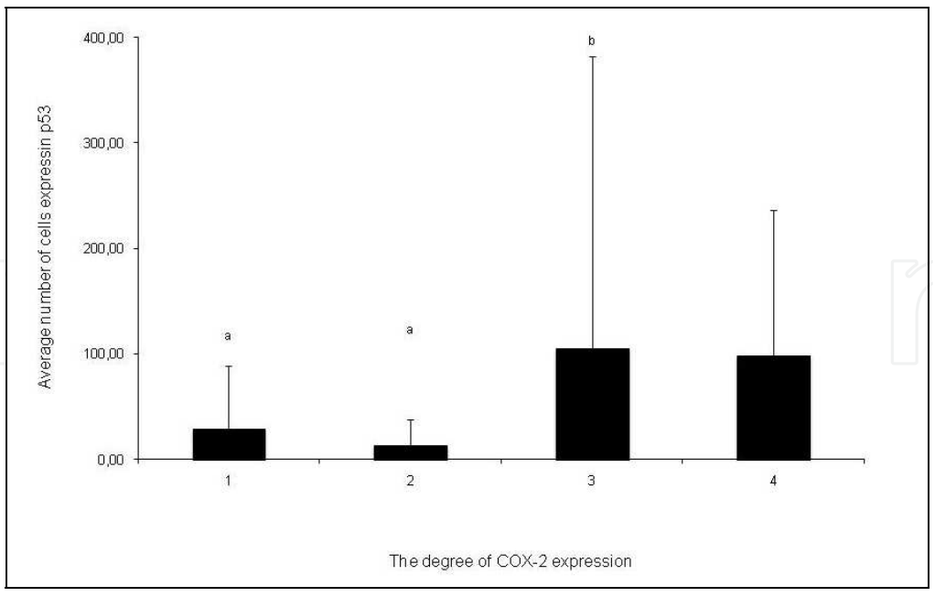


Fig. 18. Average number of cells expressing p53, depending on the degree of expression of cyclooxygenase – 2. Letters (a, b) above the columns show that the difference between means was statistically significant (P≤0.05).

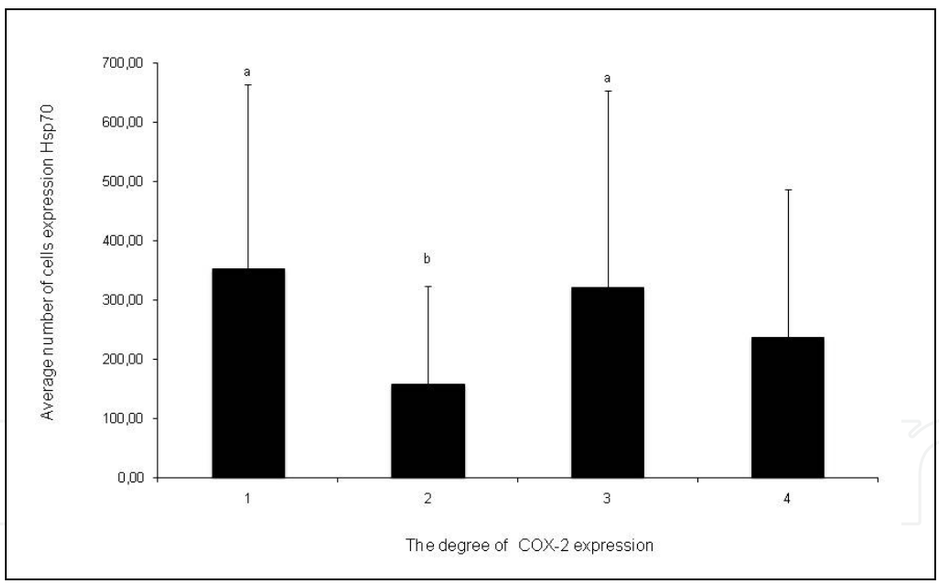


Fig. 19. Average number of cells expressing Hsp70, depending on the degree of expression of cyclooxygenase – 2. Letters (a, b) above the columns show that the difference between means was statistically significant (P≤0.05).

2.3.7 Results of P-glycoprotein expression in neoplasms of the mammary gland

Expression of P-glycoprotein was identified in cytoplasm and cell membranes of neoplastic cells. Positive reaction was found in 76% of all neoplasms. Complex carcinomas were the biggest group among cancer types, which demonstrated positive reaction to P-gp. In terms

of histological malignancy grade, the most numerous were cancers featuring the lowest grade of malignancy (Fig. 20). In female canines aged 9 through 12 years, cancers exhibiting a positive P-gp reaction constituted the most numerous group (63.2%); on the other hand, this cancer type barely appeared in the oldest group (10.5%). Analysis of the average P - glycoprotein expression in different types of carcinomas did not reveal any significant differences. Correspondingly, evaluation of malignancy grade showed no statistical significance between examined features. Among different cancer types, the highest P-gp expression was demonstrated in solid carcinomas and cancers featuring the highest histological grade of malignancy. Positive correlation between investigated cancer features was found in the analysis of the relationship between cyclooxygenase-2 and P-gp expression, where P value was equal to 0.021.

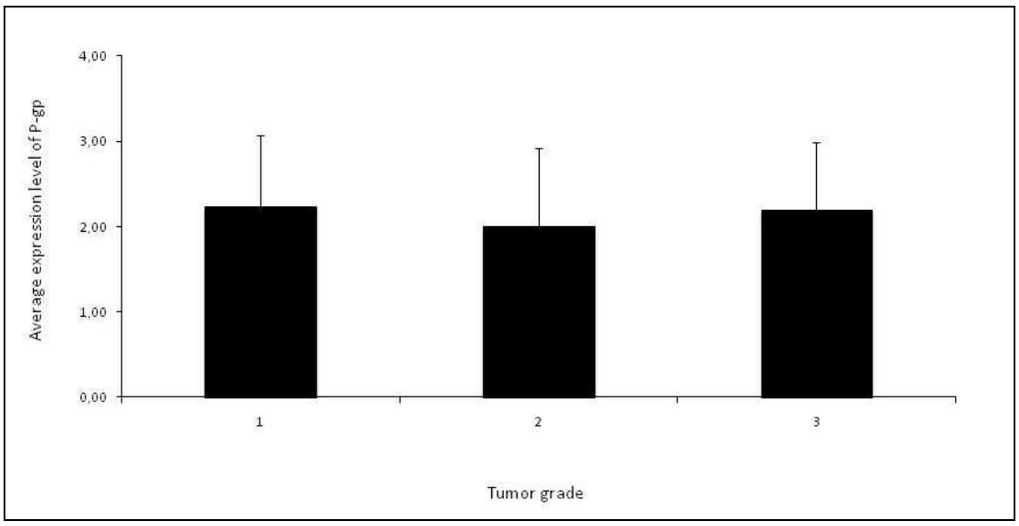


Fig. 20. Average expression level of P–gp depending on the tumor grade.

2.3.8 Results of heat shock protein expression in neoplasms of mammary gland

Heat shock proteins were found in the cytoplasm and nuclei of cancer cells (Fig. 21, Fig. 22, Fig. 23, Fig. 24). The largest group of tumors exhibiting Hsp70 and Hsp90 expression included simple and complex cancers, whereas solid tumors were the least numerous group. Grade 1 and 2 cancers constituted the largest group expressing both Hsp70 and Hsp90 (Fig. 25, Fig. 26) . Immunohistochemical analysis showed high expression of Hsp90 in simple and complex cancers, but no statistically significant differences were found between investigated types of tumors (P=0.443). High expression of Hsp90 was confirmed in solid cancers and, in this particular group, significant differences were found between types of tumor (P=0.032). As far as grading was concerned, no statistically significant differences were found between the mean number of cells exhibiting Hsp70 and Hsp90 protein expression and malignancy grade. When comparing the expression of Hsp70 to the expression of Hsp90 in particular types of cancers, we found a highly significant statistical difference (P=0.005) between the expression of both proteins. Results of a study on the expression of heat shock proteins (Hsp70 and Hsp90) were compared to nuclear

antigen Ki-67 expression. It was found that the expression of nuclear antigen Ki-67 was most pronounced in solid tumors, as was the expression of Hsp90. Also, in neoplasms of the highest grade, expression of nuclear antigen Ki-67 and protein Hsp90 was the highest. High expression of Hsp70 was found in tumors with grade 1 and 3 of cyclooxygenase-2 expression, whereas the lowest Hsp70 expression was observed in tumors with grade 2 of cyclooxygenase-2 expression. Statistical significance was found between the investigated features of neoplasms (P=0.009). Increased expression of cyclooxygenase-2 was observed in tumors with a low mean number of cells showing positive immunohistochemical reaction for protein Hsp90. The highest level of expression of this protein was confirmed in tumors with grade 1 of cyclooxygenase-2 expression (Fig. 27). Between the mean number of cells showing positive Hsp90 reaction and cyclooxygenase-2 expression, there was a statistical significance observed for grade 1 and 2 of cyclooxygenase-2 expression (P=0.039). Statistical analysis proved a correlation between Hsp70 and p53 protein expression in tumors of epithelial origin. High statistical significance was shown for investigated neoplastic features (P = 0.002). Taking into account the type of tumor, expression of both proteins was highest in complex carcinomas and tumors with the lowest histological grade. High expression levels of Hsp90 protein and Ki-67 nuclear antigen was shown in cases of solid carcinomas and in carcinomas exhibiting 3rd histological grade of malignancy, described by a low apoptotic index.

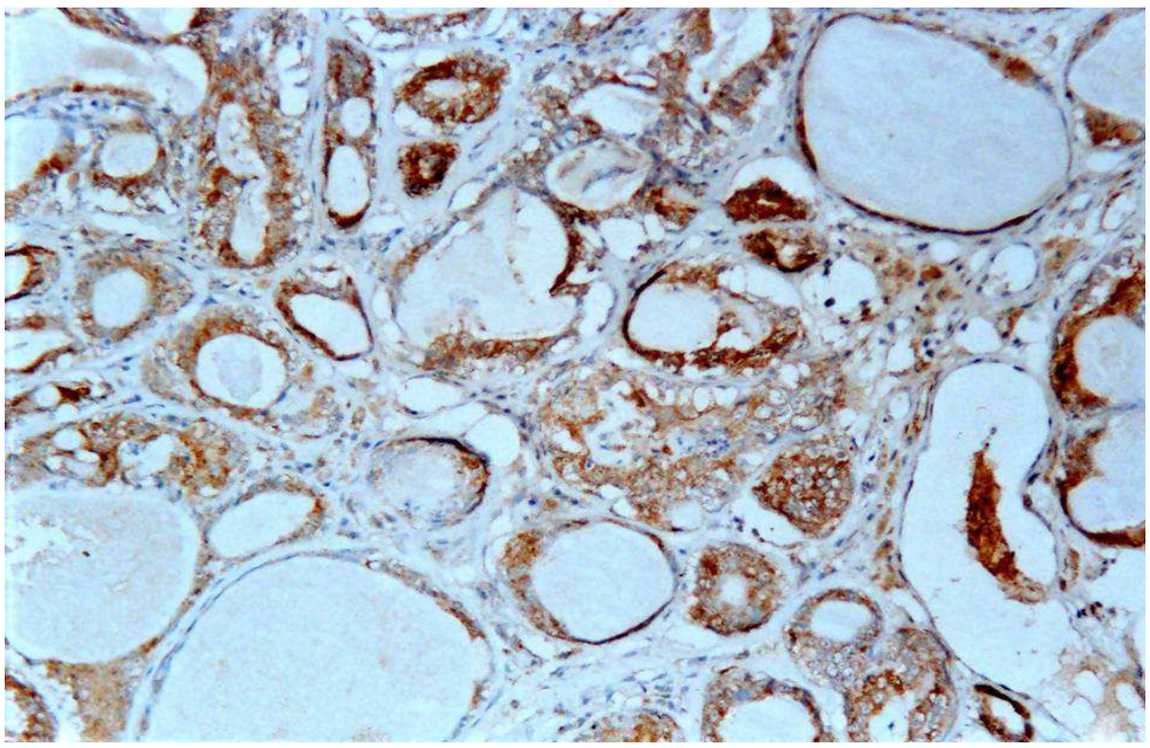


Fig. 21. Adenoma, expression of Hsp70, immunohistochemical method. (20x)

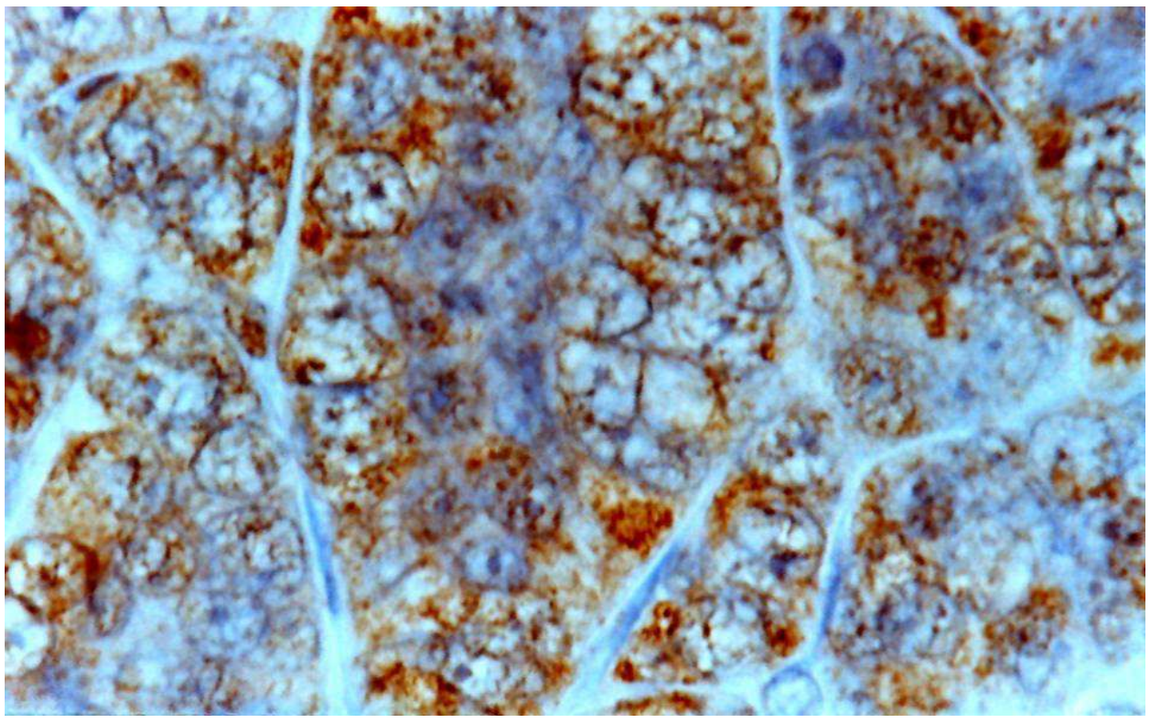


Fig. 22. Adenocarcinoma simplex, expression of Hsp70, immunohistochemical method. (100x)

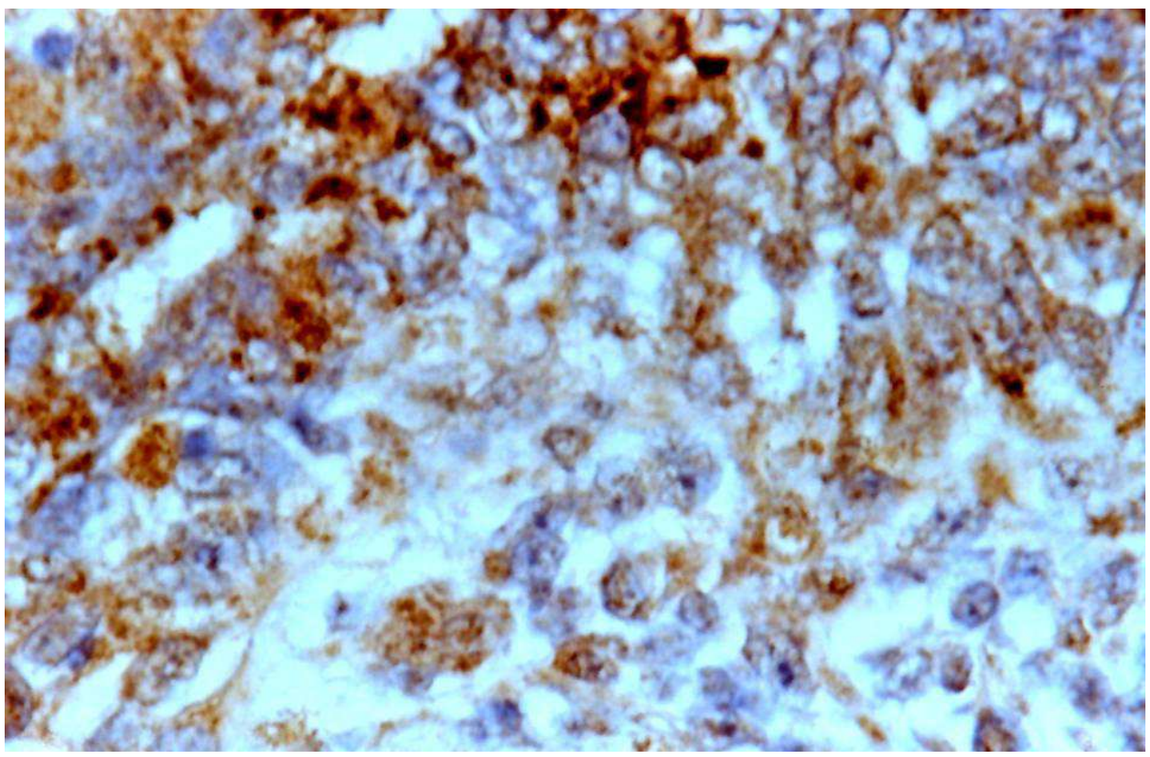


Fig. 23. Adenocarcinoma complex, expression of Hsp70, immunohistochemical method. (100x)

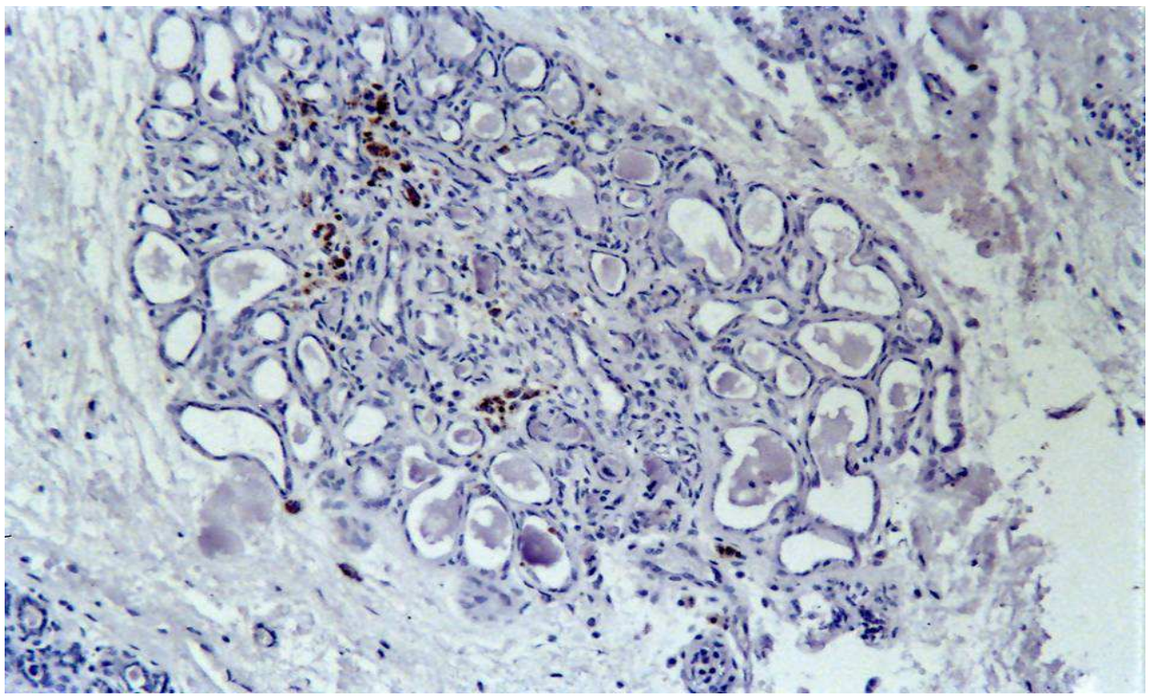


Fig. 24. Adenocarcinoma complex, expression of Hsp90, immunohistochemical method. (10x)

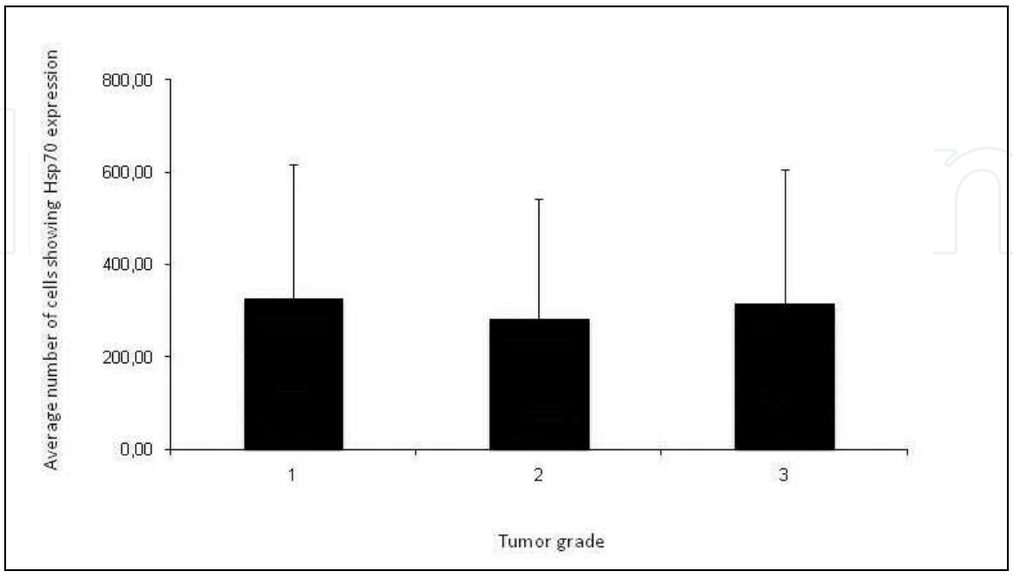


Fig. 25. Average number of cells showing Hsp70 expression depending on the tumor grade.

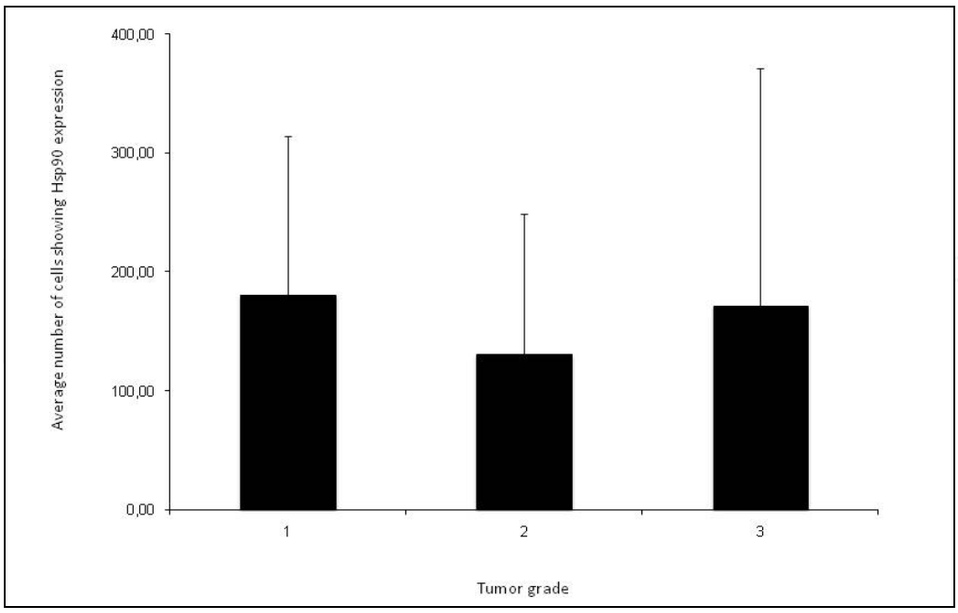


Fig. 26. Average number of cells showing Hsp90 expression depending on the tumor grade

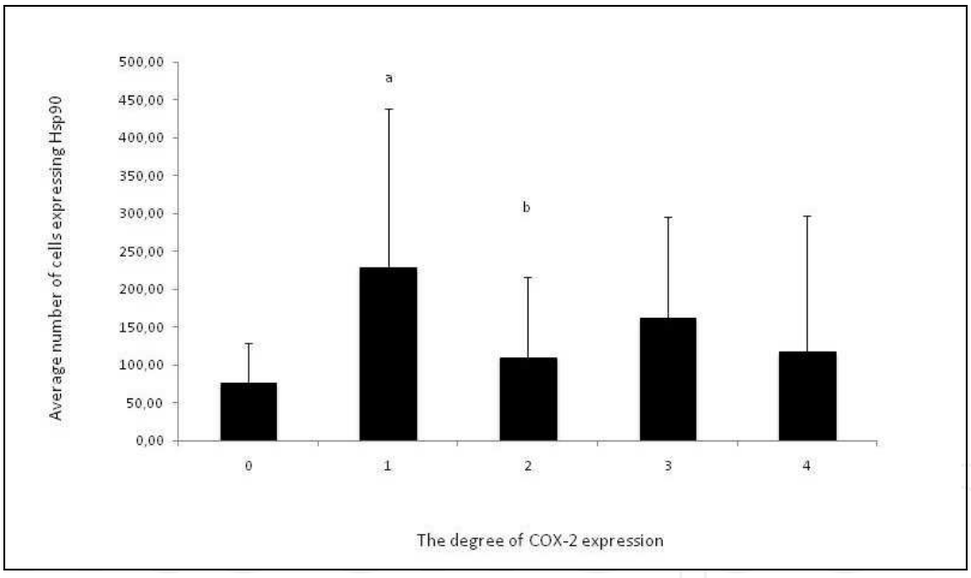


Fig. 27. Average number of cells expressing Hsp90, depending on the degree of cyclooxygenase – 2 expression. Letters (a, b) above the columns show that the difference between means was statistically significant (P≤0.05).

3. Discussion and conclusion

3.1 Discusion

In our study, cancers accounted for 89.8% of tumors of epithelial origin. This is consistent with the results of some authors, although the results of studies on the incidence and types of tumors of the mammary gland in canine females given by various authors differ.

Löhr (1997) found that 50% of the mammary gland tumors in female canines are malignant tumors (Löhr et al, 1997). Hellmén (1993) determined the incidence of malignant tumors to be 68% (Hellmén et al 1993). There are also studies showing, that about a half of tumors are benign (Bostock et al, 1992; Gilbertson et al, 1983). Moulton (1990) believes that benign tumors represent about 80% of cases and the majority of tumors studied are "benign mixed tumors" (65%) (Moulton, 1990). They are tumors in which, apart from epithelial and mesenchymal tissue, there is also cartilage and bone tissue. Their histopathological examination reveals no sign of malignancy. Nerurkar (1990) says that benign tumors represent about 27% of the mammary gland tumors in female dogs (Nerurkar et al, 1990). In our study, benign tumors of epithelial origin accounted for only 10.1% of all cases. The diversity of these results relates to the lack of uniform diagnostic criteria and lack of uniform classification of tumors of the mammary gland in dogs. In human medicine, age is a very important prognostic factor. Detection of breast cancer in women at a very young age and at the age over 60 is associated with worse prognosis (Host & Lund, 1986). In Philibert's (2003) studies there were no significant differences in survival of young and older dogs (Philibert et al, 2003). Hellmén (1993) presented completely different results (Hellmén et al, 1993). She found that age may be an important prognostic factor and showed that older bitches had a shorter survival time after surgery. Results obtained by Benjamin corresponded to that (1999) (Benjamin et al, 1999). Own results support the concept of age as an important prognostic factor, as there is very little information regarding survival or recurrence of malignancy in female dogs after surgery. Current study shows that the presence or absence of cellular inflammatory infiltrates may be a prognostic factor. Data on the incidence of cellular infiltration in mammary gland tumors in female dogs is also scarce. Gilbertson (1983) found that cell infiltration plays a role in the process of development of precancerous and invasive carcinomas (35%) (Gilbertson et al, 1983). In our study, the percentage of tumors with cellular infiltration was higher (88%). The highest intensity of cellular infiltration was observed in tumors with a high histological grade of malignancy. Skrzypczak (2004) (Skrzypczak, 2004) presented similar results in his study . Authors believe that presence of cellular infiltration plays a positive role in inhibiting tumor growth. Some studies have shown that presence of cellular infiltration is associated with good (Rilke et al, 1991) or poor (Parl & Dupont, 1982) prognosis and others, that it carries no prognostic value. In our study, no relationship was found between cellular infiltration and other tumor markers, consistent with the studies by Rodo (2007) (Rodo, 2007; Roses et al, 1982). It is worth noting the presence of necrosis in tumor foci, which may obscure the accuracy of the results. Necrosis in the tumor is the result of disparities between high proliferative activity and tumor vascularization. It may trigger cellular inflammatory reactions. We also analyzed the distribution of cellular infiltration. Our own results differ from the results of other authors, because infiltration was observed in the stroma, not scattered (Lee et al, 1996), and was more pronounced on the periphery of the tumor. The role of cellular infiltration within the tumor remains unclear and controversial. Estrogen receptors are recognized markers in the diagnosis of breast cancer in women. It is estimated that about 70-80% of breast cancers in women exhibit the expression of estrogen receptors. These tumors are characterized by slower growth, higher diversity, better prognosis with a suitable treatment regimen and correlate with the length of survival after

surgical removal (Bacus et al, 1989; Barzanti et al, 2000). Study of the expression of estrogen receptors in mammary gland neoplasms in female canines was, as usual, inconclusive. Martin et. al (1984) studied 228 tumors of the mammary gland in bitches and showed the expression of estrogen receptors in only 2.1% of tumors (Martin et al, 1984). Pena et. al (1998), on the other hand, diagnosed 21 cancers with an ongoing inflammatory process and found no expression of estrogen receptors (Pena et al, 1998). According to Sartin et. al (1992) the greatest chance of long-term survival after surgery , is associated with tumors expressing ER alone or together with PgR. Indeed, in the absence of ER and PgR, researchers observed the shortest period of survival (Sartin et al, 1997). Millanta et. al (2005) study on 47 mammary gland neoplasms in bitches reported that ER and PgR expression did not correlate with survival or histological parameters of tumors (Millanta et al, 2005). Similarly, Sobczak-Filipiak and Malicka (1997) have shown no correlation between expression of ER and mitotic index (Sobczak-Filipiak & Malicka, 1997). In our study, the expression of estrogen receptors was demonstrated in 40% of the tested tumors. The highest expression of estrogen receptors was found in simple carcinomas and tumors with the highest histological grade of malignancy. There was a significant correlation between mitotic index and expression of estrogen receptors. Nowak (2007) found expression of estrogen receptors in only 6% of cancers (Nowak et al, 2007), but Mulas (2005) obtained different results- he found that estrogen receptors were expressed in benign tumors (Mulas et al, 2005). Similarly, McEwen et. al (1982) have demonstrated the expression of estrogen receptors in about 50% of cases, with a significantly higher levels of expression present in benign tumors (McEwen et al, 1982). Results obtained by Nieto et. al (2000) are different - they show a correlation between the expression of nuclear antigen Ki-67 and estrogen receptors (Nieto et al, 2000). Authors of these studies observed the highest levels of ER expression in simple and complex carcinomas, as we did in our study. Otherwise, Rutteman (1998), Sartin (1992), and Geraldes (2000), claim that high expression of ER is present in adenomas compared to adenocarcinomas, which exhibit a lower level of expression of this marker (Sartin et al, 1992; Rutteman et al, 2001; Geraldes et al, 2000). Opinions about the value of estrogen receptors as a prognostic factor are divided. Some authors consider ER expression a positive factor, but there are also voices postulating it is a negative prognostic marker. Interestingly, it seems that in our study, low expression of ER negatively correlated with high expression of nuclear antigen Ki-67. It is in agreement with the research done by Peña (1998), who led the study on the mammary gland neoplasms in canines (Peña et al, 1998). From our research we conclude, that the expression of estrogen receptors may be important in assessment of malignancy, but does not show significant correlation with other markers. An important marker of malignancy is the proliferative activity. Proper evaluation of proliferative activity of tumor cells is crucial for the evaluation of its biological activity and is used in determining the treatment of cancer. High mitotic index correlated with tumor size and presence of lymph node metastases (Niwińska, 1995; Mirecka et al, 1993). In our study, proliferative activity depended on both, the type of tumor and the degree of histological malignancy. The highest values of mitotic index were recorded in simple and solid carcinomas and in tumors with the highest histological grade of malignancy. Similar results were reported for the expression of Ki-67. The highest expression of Ki-67 was seen in solid, simple carcinomas and in 3rd grade tumors. Similar results were obtained by Nieto (2000), who

confirmed that the expression of Ki-67 is high in tumors with a higher histological grade of malignancy (Nieto et al, 2000). Similar results were obtained by Peña (1998), Giziński (2003) and Szczubiał (2002) (Peña et al, 1998; Giziński et al, 2003; Szczubiał & Łopuszański, 2002). They studied the expression of nuclear antigen Ki-67 in mammary gland tumors in dogs and came to the conclusion that higher expression of Ki-67 is an important prognostic factor and is associated with a higher risk of metastasis, a shorter period to recurrence and a shorter overall survival period. The rate of tumor growth is influenced by factors related to inhibition of cell cycle and promotion of apoptosis of cancer cells., Under physiological conditions it is a function of, among others, p53 gene and its protein product. Mutation of the p53 gene plays an important role in the uncontrolled proliferation and resistance to apoptosis in cancer cells , leading to functional changes in proteins. In dogs, expression of the p53 gene was found in both benign (Muto et al, 2000) and malignant (Mayr et al, 1994; Veldhoen et al, 1999) tumors of mammary gland. Muto (2000) in his study showed no association between p53 expression and histological type of cancer, but claimed that this protein may play an important role in carcinogenesis and may be a negative prognostic factor (Muto et al, 2000). Chung-Ho (2004) came to a similar conclusion, arguing that the presence ofp53 in tumor cells is associated with their malignancy and bad prognosis in mammary gland tumors (Chun-Ho et al, 2004). In our study, p53 expression was observed in benign as well as malignant tumors. Among all tumors, more than 50% exhibited a positive reaction for 53 protein. It contradicts the data obtained in his study by Gamblin (1997), who carried out an analysis of 16 adenocarcinomas and found a positive reaction for p53 protein in only 12.5% of cases (Gamblin et al, 1994). In our study, the highest expression of p53 was observed in complex carcinomas and in 1st and 2nd grade malignant tumors. These results are consistent with the results obtained by Rungsipipata (1999), who found the highest levels of p53 expression in simple and complex carcinomas and also showed expression of this protein in adenomas (16%) (Rungspipata, 1999). Rodo (2007) obtained results on the expression of p53 , stating that there is a positive correlation between proliferative activity and the number of cells expressing p53 protein (Rodo, 2007). In our study, we found no statistically significant differences between the expression of p53 and nuclear antigen Ki-67. Obtained data in part explain why the activation of oncogenes does not always lead to uncontrolled proliferation when normal signal transduction leads to stabilization of p53 and activation of programmed cell death. There is a need for further research in this area, because the data in the literature is ambiguous and does not allow for a definite conclusion as to the importance of p53 protein and its role in mammary gland neoplasms. Varying results undermine the claim that a positive reaction for p53 protein would predict worse prognosis in cancer and that p53 mutation may be responsible for increased proliferation in tumors with advanced malignancy. Research on COX-2 expression in canine mammary cancers is scarce (Heller et al, 2005; Nowak et al, 2005; Doré et al, 2003) despite it being an attractive and motivating topic. Overexpression of COX-2 is known to occur in 56-100% of canine mammary carcinomas (Doré et al, 2003; Millanta et al, 2005; Queiroga et al, 2007; Heller et al, 2005; Mohammed et al, 2004), but there is a marked variation in the percentage of COX-2-positive tumor cells and the intensity of its expression. Doré et. al (2003) did not find any COX-2 expression in four samples of normal mammary tissue (Doré et al, 2003), whilst Mohammed et. al (2004) (Mohammed et

al, 2004) and Queiroga et. al (2007) (Queiroga et al, 2007) reported expression of COX-2 in one of seven and two of four samples of normal mammary glands, respectively. Our own research demonstrated that as much as 91.7% of all carcinomas in the study displayed COX-2 expression. Doré et.al (2003) obtained similar results: he found a positive COX-2 reaction in 67% of complex cancers and in 47% of simple carcinomas (Doré et al, 2003). Our research demonstrated the highest COX-2 expression in simple cancers and the lowest in solid cancers. Similar data were presented by Heller et.al (2005), who found COX-2 expression in adenocarcinomas, whereas he failed to detect it in solid carcinomas (Heller et al, 2005). Ristimäki et. al (2002) demonstrated the relationship between COX-2 expression and certain clinical and pathological features of the tumor (Ristimäki et al, 2002). Her research into mammary gland tumours proved that COX-2 expression was positively correlated with tumour size as well as the strength of Ki-67 nuclear antigen staining and p53 protein expression. Results of our study are consistent with the results of works by Ristimäki et. al (2002) : increased COX-2 expression in carcinomas is related to higher mitotic index, i.e. with the proliferating activity (Ristimäki et al, 2002). Expression of Ki–67 nuclear antigen was demonstrated in carcinomas exhibiting increased COX-2 expression, yet research failed to prove the correlation between these markers; On the other hand, it proved the existence of statistical correlation between COX-2 expression and expression of p53 and Hsp70 proteins. Average number of cells exhibiting p53 protein expression was higher in carcinomas with higher levels of COX-2 expression. High expression level of Hsp70 protein and COX-2 was shown in carcinomas with the 3rd histological grade of malignancy, as described by low apoptotic index. Similar results were obtained by Lanza-Jacoby et. al (2004), who conducted her studies on experimental animals (Lanza-Jacoby et al, 2006), and by Liu and Rose (1996) who studied the COX-2 expression in cell cultures (Liu & Rose, 1996). Furthermore, Ristimäki et al (2002) and Dempke et al (2001) came to a conclusion that COX-2 promoted the mammary gland neoplasm growth, invasive capacity, and probability of metastasis (Ristimäki et al, 2002; Dempke et al, 2001). COX-2 expression may be relevant to a number of physiological processes within this tissue including proliferative activity, inhibition of apoptosis, increased angiogenesis and activation of matrix metalloproteinases (Dempke et al, 2001). Mammary physiology is a complex process regulated by hormones, estrogens, progesterone, growth hormone, prolactin, and epidermal growth factor (Howlin et al, 2006). As COX-2 expression is induced by different stimuli including cytokines, oncogenes, hormones and growth factors (Thomas et al, 2008), the same factors that control mammary growth and differentiation may act as trigger stimuli for COX-2 expression. The results of our work are consistent with the results obtained by other researchers. P-gp expression was discovered in epithelium and neoplasms of mesenchymal origin in dogs (Ginn, 1996). Author of these studies argues that continuation of this line of research will deliver additional prognostic data. There is a small number of works attempting to evaluate the P-gp expression in canine mammary cancers. A considerable progress in the investigation into P-gp expression in canine mammary cancers was made by Petterino et. al (2006), who attempted to define the P-gp expression in mammary gland carcinomas in female canines (Petterino et al, 2006). His study covered cases of both malignant and benign neoplasms; Petterino et al (2006) found the expression of the examined marker in two test groups and confirmed the statistical

significance between the two groups (Petterino et al, 2006). Furthermore, he found that simple and complex carcinomas were the most numerous groups among the examined cancers and that P-gp expression was absent from the tissues of healthy mammary glands. We achieved similar results in our research: we found the P-gp expression in 76% of the examined canine mammary cancers. The most numerous groups exhibiting a positive P-gp immunohistochemical reaction were composed of complex carcinomas (90.9%) and simple carcinomas (73%). High expression was found in cancers with the highest histological grade of malignancy. Due to fact that there are hardly any works investigating into the P-gp expression in canine mammary cancers, we attempted not only to confirm the presence of P-gp expression and location, but also to prove the relationship between P-gp and other neoplastic markers. Statistical analysis confirmed a positive correlation between P-gp and COX-2 expression: it demonstrated a statistical significance in the case of examined characteristics ($P = 0.021$). It is worth mentioning that the expression of two markers was significant in carcinomas featuring high histological grades of malignancy. Furthermore, P-gp expression was studied in canine lymphomas; additionally, attempts were made to conduct studies on carcinomas treated by means of chemotherapy; the results were compared with the results obtained in the control group of untreated carcinomas. Higher expression was observed in the treated cancer cells. On the other hand, increased expression in untreated cancers appears to be a negative prognostic factor, given that survival rate shall decrease in such cases (Lee et al, 1996). Due to the fact that this line of research has not brought about many works, there are several questions and ambiguities concerning the role of P-gp in the process of neoplastic genesis and the importance of this factor from the clinical point of view. Additionally, the question arises whether, or not P-gp can be classified into the significant prognostic marker category. We are not aware of defensive mechanisms of the cells equipped with membrane transporters against apoptosis generated by the compounds, that are not pump substrates. There are suggestions that the phenomenon may be connected with evacuation (pumping out) of a certain important mediator of apoptosis or with the impact of P-gp on the intracellular pH (Johnson et al, 1998). Identification of a mechanism of cell resistance to apoptosis is a key instrument in selection of suitable therapy. According to studies in humans, heat shock proteins may be important predictors of breast cancer (Park & Dupont, 1982). There is not much published data in the literature on heat shock protein expression in mammary gland tumors of female dogs. The role of these proteins in carcinogenesis has not been clearly defined either. Studies have only shown that expression of heat shock proteins takes place in canine breast cancers , but the linkage between these proteins and other tumor markers was not confirmed. Seymour (1990) studied endometrial cancer in women and found that heat shock proteins were useful markers in the diagnostics of these tumors (Saymour et al, 1990). Expression of Hsp27 was also investigated in breast cancer in women (Ciocca et al, 1993). A relationship between the expression of this protein and the degree of differentiation of the tumor cells was found. Similar studies were conducted by Storm (1996), who stated that tumors with Hsp27 expression showed a higher histological grade than tumors negative for the expression of this protein (Storm et al, 1993). Kumaraguruparan (2006) studied the expression of Hsp70 and Hsp90 in breast cancer in women and found a correlation between the expression of both proteins and proliferative activity (Kumaraguruparan et

al, 2006). He suggested that heat shock proteins are important prognostic factors. Presence of Hsp70 was also reported in normal gastrointestinal tissues, but an increased expression of this protein was observed in gastrointestinal tumors (Isomoto et al, 2003). Isomoto (2003) believed that Hsp70 plays an important role in the degree of cellular differentiation in tumors, which would indicate that Hsp70 is an important prognostic factor. Similar studies are conducted in veterinary medicine, but to a lesser extent (Isomoto et al, 2003). A similar study was conducted by Rommanucci (2005) in mammary gland tumors in female dogs (Romanucci et al, 2006). She found Hsp70 and Hsp90 expression in simple and complex, as well as solid-type adenocarcinomas. Expression of these proteins was observed in the cytoplasm, as well as in the nuclei of tumor cells. Rommanucci (2006), in her study of breast cancers in female dogs, has not attempted to verify the possible correlations between heat shock proteins and other tumor markers (Rommanucci et al, 2006). She focused on establishing the degree of protein expression and their localization in tumor cells. Basing on the results of her study, the author suggested that these proteins may play an important role in the process of carcinogenesis. In our study, Hsp70 protein expression was found in 86.4% of tumors, while the expression of Hsp90 was observed in 66.2% of tumors. The aim of our study was to demonstrate the relationship between the expression of heat shock proteins and other prognostic factors. When comparing the expression of Hsp70 to the expression of Hsp90 in different types of tumors, we found a high statistical significance between the expression of both proteins. Analysis of the relationship between the expression of nuclear antigen Ki-67 and the expression of heat shock proteins showed that the highest expression of Ki-67 as well as Hsp90 was present in solid tumors. The highest expression of Hsp90 protein and Ki-67 was also found in cancers of the highest histological grades. Based on these data it can be concluded that Hsp90 is an important factor, which could be considered a marker of malignancy and which may be useful in the diagnostics of cancers. Analysis of the results showed high expression of Hsp70 in cancers with grade 1 and 3 of COX-2 expression; the lowest expression of Hsp70 was found in cancers with grade 2 of COX-2 expression. Statistical significance of $P = 0.009$ was found between the expression of Hsp70 and expression of COX-2. An increased expression of COX-2 was observed in tumors with a low mean number of cells showing positive reaction to Hsp90 protein. Correlation between the expression of Hsp70 protein and p53 protein in tumors of epithelial origin was also confirmed. Based on the analysis of expression of both proteins and taking into account the mean apoptotic index, it was found that in cancers with the third (the highest) histological grade, expression of p53 protein is significantly lower than the expression of Hsp70, and the value of apoptotic index in these cancers was the lowest.

3.2 Conclusions

Occurrence of mammary tumors in female dogs and their potential use as a model in comparative pathology makes it an interesting research material. It is known that the degree of malignancy is positively correlated with proliferative activity. The relationship between expression of estrogen receptors and the degree of malignancy is not entirely clear, although most authors believe that the expression of these receptors is higher in benign tumors.

Literature concerning the expression of p53 protein in mammary tumors in canines is scarce. It deals only with the expression of p53, but leaves no answers in respect to the expression of other factors that are considered prognostic markers, such as proliferative activity, histological grade of malignancy and expression of estrogen receptors. We conclude that COX-2 is an important prognostic factor and may be applied as a marker of canine mammary neoplasm malignancy, given the fact that higher expression of COX-2 was found in adenocarcinomas and in cancers featuring the highest histological malignancy grades in comparison to simple adenomas. Obtained results suggest that cyclooxygenase-2 may be a prognostic factor, but it requires clinical confirmation. The P-gp expression is also positively correlated with the degree of histological malignancy. This suggests a prudent approach to decisions concerning chemotherapy. Expression of Hsp90 can be considered as a marker of the degree of differentiation and of histological grade of mammary gland tumors in dogs, because the highest level of expression was found in solid carcinomas and in cancers exhibiting the highest histological grades of malignancy. Hsp70 expression was confirmed, but no correlation with other factors was found. This may suggest that Hsp70 is not a useful marker in the diagnostics of breast cancers in female dogs.

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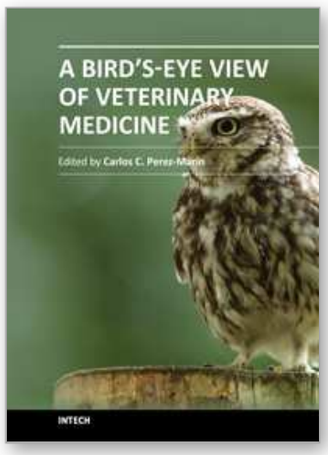
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Veterinary medicine is advancing at a very rapid pace, particularly given the breadth of the discipline. This book examines new developments covering a wide range of issues from health and welfare in livestock, pets, and wild animals to public health supervision and biomedical research. As well as containing reviews offering fresh insight into specific issues, this book includes a selection of scientific articles which help to chart the advance of this science. The book is divided into several sections. The opening chapters cover the veterinary profession and veterinary science in general, while later chapters look at specific aspects of applied veterinary medicine in pets and in livestock. Finally, research papers are grouped by specialisms with a view to exploring progress in areas such as organ transplantation, therapeutic use of natural substances, and the use of new diagnostic techniques for disease control. This book was produced during World Veterinary Year 2011, which marked the 250th anniversary of the veterinary profession. It provides a fittingly concise and enjoyable overview of the whole science of veterinary medicine.

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