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EVALUATION OF COX-2 EXPRESSION IN CANINE MAMMARY TUMORS

AND ITS RELATION TO NSAID THERAPY

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2013

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1.INTRODUCTION AND LITERATURE BASED REVIEW

1.1 Mammary gland neoplasms; their incidence and clinical signs.

Mammary gland tumors are common in the dog, less common and often more aggressive in the cat, but rare in other domestic animals (Munson and Moresco, 2007). It has been long known that mammary gland tumors are one of the most common neoplasms in intact female dogs (Rivera et al., 2009). Mammary gland tumors may also affect male dogs in rare cases, with the prognosis being more guarded in them (Saba et al., 2007).

In a typical female dog there are 5 pairs of mammary glands, beginning on the axilla and extending to the inguinal area, with each pair on either side of the ventral midline. The nipples indicate their location on the trunk of the body, with two thoracic, two abdominal, and one inguinal pair of mammary glands (Sorenmo et al., 2011).The largest of these are the inguinal mammary glands and also the most susceptible ones to neoplasia (Baba and Câtoi, 2007).

Most mammary gland tumors occur in dogs over 6 years of age, the average age of incidence being about 10 years (Alenza et al., 2000), and about half of them are malignant. However, if they occur in a dog below two years of age, they are rather usually benign. Multiple tumors, affecting more than one mammary gland, are common as well. Other risk factors for canine mammary neoplasia include hormonal imbalance, breed and familial predisposition, obesity, diet, spaying, pregnancy, lactation and early weaning (Rivera et al., 2009).

The principal clinical sign associated with tumor growth in the mammary gland is the appearance of a painless lump or mass, which continues to grow slowly or rapidly over time. This mass can be small or large, fixed or freely movable, and single or multiple (Sorenmo et al., 2011). Occasionally, the mass ulcerates and bleeds. Canine mammary neoplasia is a heterogenous and complex group of epithelial, mesenchymal and mixed tumors that show variable clinical features from benign to highly malignant (Goldschmidt et al., 2011). Therefore, individualized care and therapy is required to treat them, besides simply depending on their clinical staging or grading for therapeutic interventions.

Malignant tumors often metastasize primarily to the regional lymph nodes (draining the respective mammary glands), lungs, liver and bones, therefore examination by chest

radiography, abdominal ultrasound, CT scanning, FNAB and core needle biopsies is routinely helpful in detecting metastases (Cassali et al., 2011).

Canine mammary tumor is considered as an excellent naturally occurring model for human breast cancer as both show vast similarities in clinical behaviour and outcome, and bio-molecular pattern and genetics. In human and veterinary medicine, there is a constant search for prognostic factors that allow for precise evaluation of the state, survival, treatability and risk of re-occurence of these tumors. Besides the basic histopathological examination that helps differentiate various types of cancers, additional information provided by research on cancer biology is by the the study of tumor markers (Kozakiewicz, 2012).

1.1.1 Prognostic factors in mammary gland tumors.

Specific clinical, pathological and biological characteristics of individuals and their tumors that permit prediction of clinical outcome and survival are defined as prognostic factors. With respect to canine mammary tumors, these factors are useful for studies concerning comparative pathology, and the search for novel avenues of diagnostic, prognostic and predictive value. The age of the animal, sentinel lymph node status, histological sub-type of tumor and grade of malignancy, as well as mitotic index assesment can serve as prognostic factors to predict the behavior and clinical outcome of mammary tumors.

1.1.2 Tumor markers; their definition and use.

Molecular markers which have been evaluated as information sources for prognosis, to predict the behavior of various types of cancers in humans and animals are termed as tumor markers. In simple words, any molecular species that is produced in abnormal amounts or under abnormal circumstances by a tumour, may be useful as a tumour marker. Most tumor markers are proteins, but patterns of gene expression and complex changes in DNA or epigenetics have also been used as tumor markers in the recent years. Some are associated with only one type of cancer, whereas, others with two or more cancer types but there is no universal tumor marker that can detect any type of cancer (Bigbee & Herberman, 2003).

Many serum tumor markers have been evaluated as a tool for cancer diagnosis, prognosis, staging, treatment monitoring, and response to therapy. They have also been evaluated as

prognostic factors, either alone, or in combination with other histopathological, biochemical and clinical variables (Ferrigno et al., 1994).

Some examples of important prognostic markers evaluated in canine mammary tumors are hormone receptors (estrogen receptor - ER and progesterone receptor - PR), a marker of the tumor proliferative index (MIB-1), a marker of angiogenesis (CD31), adhesion molecules (E-cadherin and β-catenin), epidermal growth factor (EGF), p53 and HER-2 oncogene (Cassali et al., 2011). The aim of research on the mammary gland neoplasms in female dogs is to extend the existing panel of tumor markers by adding newer ones like P-gp, Hsp90, Hsp70 and COX-2, and to incorporate them into routine diagnostics (Kozakiewicz, 2012).

1.2 Cyclo-oxygenase enzyme; its isoforms.

One such marker is the COX-2, a key enzyme in eicosanoid biosynthesis. It is a rate limiting factor in the synthesis of prostaglandin from arachidonic acid. Two isoforms of COX are known, COX-1, the constitutive isoform; and COX-2, the inducible isoform of the enzyme. Studies in human breast cancer patients revealed that COX-1 is expressed ubiquitously, and its role is connected to physiological functions, such as cytoprotection of the stomach and control of platelet aggregation (Ristimäki et al., 2002). Whereas the role of COX-2, the inducible form of the enzyme, has been connected to inflammation and carcinogenesis. COX-2 is usually undetectable in normal tissues, but undergoes rapid induction in response to cell activation by pro-inflammatory cytokines, growth factors, oncogenes, bacterial lipopolysaccharides and phorbol esters. Thus COX-2 is overexpressed in some malignancies and premalignant lesions and not COX-1 (Howe, 2007).

COX-2 is a key enzyme that controls the conversion of arachidonic acid to prostaglandin. The precursor of PG synthesis is arachidonic acid, a 20 carbon polyunsaturated fatty acid. The hydrolysis of phospholipids to produce free arachidonic acid is catalysed by phospholipase A2. The next step which inserts molecular oxygen into arachidonic acid is catalysed by COX-2. This reaction produces an unstable product, PGG2, which is then converted by the peroxidase activity of COX to PGH2. PGH2 is the common precursor for all other prostanoids. The production of individual prostanoids is catalysed by different, specific synthases, which may vary in their expression between different types of cells. Each of the products derived from PGH2 has a distinct biological function (Davies et al., 2002).

1.2.1 COX-2 overexpression and carcinogenesis.

The relation between COX-2 and cancer has been studied for many years. This relationship has been postulated based on various studies which established an association between chronic use of nonsteroidal anti-inflammatory (NSAID) drugs and decreased incidence of colorectal carcinoma shown first in human patients (Thun et al., 1993), where the role of COX-2 and prostaglandins was suspected after epidemiological studies had revealed that the regular intake of low doses of aspirin reduced the risk of colorectal cancer.

As in colorectal cancer, elevated expression of COX-2 has been reported in breast cancer in women with studies showing that approximately 40 to 50% of invasive breast carcinomas have high COX-2 protein levels. Carcinomas with increased COX-2 expression have been correlated with worse prognosis for women with breast cancer In addition to colorectal cancer, many other types of human malignancies have now been shown to overexpress COX-2, such as breast, pulmonary, head and neck, pancreatic, prostatic, and gastric cancers (Doré, 2011).

Based on several experimental studies, mechanisms associated with tumoral promotion such as an increase in angiogenesis, inhibition of apoptosis, suppression of the immune response and acquisition of greater invasion capacity and metastasis modulation of immune response, and greater invasive and metastatic capacities have been proposed to explain the consequences of COX-2 overexpression (Howe, 2007).

1.2.2 COX-2 overexpression and its relation to canine mammary carcinogenesis.

In veterinary medicine COX-2 is shown to be overexpressed in a variety of tumors including tumor of the prostate, transitinal cell carcinoma, mammary gland tumors, skin and nasal tumors, among others (Queiroga, 2008). In a study in the year 2003, it was demonstrated for the first time that COX-2 is induced in a proportion of canine mammary tumors suggesting a potential role for COX-2 in canine mammary tumorigenesis (Doré et al., 2003). Malignant canine mammary tumors express COX-2 more strongly than benign tumors, irrespective of histologic type (Lavalle et al., 2009). About 60% of canine mammary gland tumors show COX-2 overexpression with most of them being malignant. Hence, the higher the expression of COX-2, the poorer the prognosis (Queiroga, 2008).

1.2.3 COX-2 overexpression and its role in angiogenesis.

It had been suggested over a decade ago, that COX-2 overexpression may lead to increased angiogenesis, such that the inhibition of COX-2 might have a general anti-cancer effect via decreased blood vessel formation in humans (Davies et al., 2002). This link between COX- 2 overexpression and tumor angiogenesis was established by various studies in human medicine in the following years (Basu et al., 2006), but more recently, Queiroga found a sound correlation between tumor angiogenesis and COX-2 overexpression in canine mammary cancer (Queiroga et al., 2010).

COX-2 is associated with the production of vascular endothelial growth factor (VEGF), which stimulates the growth of endothelial cells, and thereby, promotes angiogenesis, which is needed for most solid tumors as newly formed blood vessels provide nutrients for their growth and survival (Nardi et al., 2011). The use of COX-2 specific inhibitors has been suggested as a potential therapeutic tool for canine mammary gland tumor management by blocking COX-2 mediated angiogenesis pathways (Queiroga et al., 2011).

1.2.4 COX-2 overexpression and apoptosis inhibition.

The inhibition of apoptosis, a process of programmed cell death, appears to be the key pathway in the survival of cancer cells. COX-2 plays an important role in tumor cell biology acting through apoptosis inibition, by promoting invasiveness and by modulating the production of variable angiogenic factors. It has been postulated since long that mechanisms underlying the association between COX-2 overexpression and tumorigenic potential may include resistance to programmed cell death (Crofford, 1997).

The role of COX-2 in apoptosis has been observed in rat intestinal epithelial (RIE) cells. Studies suggest that COX-2 overexpression inhibits apoptosis by the induction of Bcl-2 expression or other members of the Bcl family (Mcl1) and that this anti-apoptotic effect can extend the survival of abnormal cells. The upregulation of Bcl-2 in tumor microvascular endothelial cells not only enhances cell survival, improving the ability of these cells to remain viable and functional despite the constraints imposed by the tumor microenvironment, but also engages them in a more vigorous angiogenic response in vitro and in vivo (Harmey, 2004). The fact that tumors derived from cells that overexpress Bcl-2 grow more aggressively

in vivo has been attributed by several authors to the anti-apoptotic properties of Bcl-2 resulting in neoplastic transformations. Also there is evidence of reduction of Bcl-2 expression and induction of apoptosis in cancer cells after the use of COX-2 inhibitors (Cao and Prescott, 2002).

1.3 NSAIDs; mechanism of action

NSAIDs are a heterogeneous group of compounds that are often chemically unrelated but have similar mechanisms of action. They may be grouped as salicylates (aspirin), arylalkanoic acids (diclofenac, indomethacin, nabumetone, sulindac), 2-arylpropionic acids or profens (ibuprofen, carprofen, ketoprofen, naproxen), *N*-arylanthranilic acids or fenamic acids (mefenamic acid, meclofenamic acid), oxicams (piroxicam, meloxicam), pyrazolidine derivates (phenylbutazone), sulfonanilides (nimesulide), and others. They are the most widely used drugs to treat pain and inflammation. The efficacy of NSAIDs may vary by patient and by indication, so substitution of an ineffective NSAID with another from a different class seems a logical option (Meek et al, 2010).

Targeting inflammatory states with non-steroidal anti-inflammatory drugs (NSAID'S) have found widespread application with increasing interest focused on their employment for cancer prevention as they are the inhibitors of the two isoforms of cyclooxygenase (COX) or prostaglanding G/H synthase. Several preclinical and clinical studies have clearly shown a reduction in the risk of cancer with the use of nonsteroidal anti-inflammatory drugs.

There are two main groups of NSAIDs: Traditional NSAIDs that inhibit both COX-1 and COX-2 (e.g. diclofenac, indomethacin, piroxicam), and Coxibs, more commonly known as the specific COX-2 inhibitors (e.g. celecoxib, meloxicam, firocoxib). The latter exclusively bind COX-2, which results in fewer gastrointestinal side effects (Queiroga *et al*, 2010). Hence, particular attention is now being drawn to specific inhibitors of the inducible COX-2 rather than the constitutive COX-1 isoform.

Both tNSAIDs and Coxibs inhibit PG biosynthesis. One of the PGs produced at high levels in the tumor microenvironment is PGE2. A better understanding of PGE2 signaling could enable identification of novel and safer therapeutic targets downstream of the cyclooxygenase enzymes. Recent studies are aimed at the emerging molecular mechanisms by which COX-2-

derived PGE2 is involved in cancer progression and delineate potential opportunities for development of novel pharmacologic approaches using this pathway (Cha and DuBios, 2007).

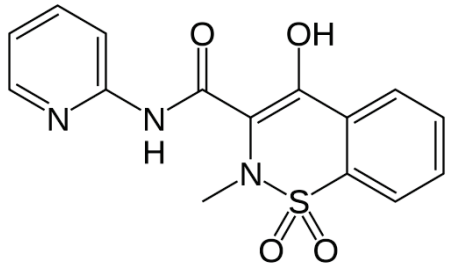


Fig.1. Chemical structure of piroxicam.

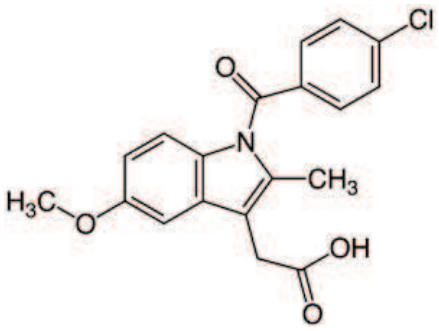


Fig.2. Chemical structure of indomethacin.

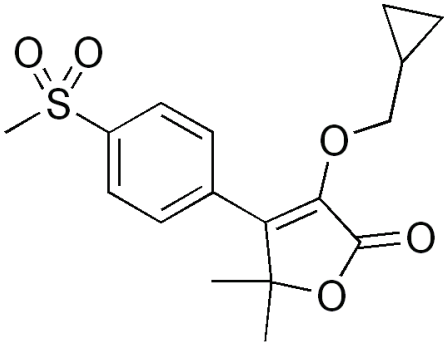


Fig.3. Chemical structure of firocoxib.

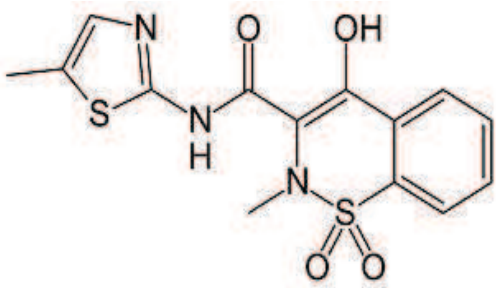


Fig.4. Chemical structure of meloxicam.

NSAIDs block enzymes in the body that help make prostaglandins, chemicals that play a role in pain and inflammation. The mechanism of action of NSAIDs can be divided into their effects on inflammation, pain, and fever expressed as the 4 A's i.e. Anti-inflammatory, Analgesic, Anti-pyretic and Anti-thrombotic effects.

NSAIDs exert their anti-inflammatory effect through inhibition of PGG/PGH synthase, or cyclooxygenase. Activation of endothelial cells and expression of cell adhesion molecules are believed to play a role in targeting circulating cells to inflammatory sites. NSAIDs may inhibit

expression of these cell adhesion molecules and may directly inhibit activation and function of neutrophils.

NSAIDs are listed as potent mild analgesics, the reason being that pain mediated by inflammation is much more likely to be relieved by NSAIDs than pain that is unrelated to inflammation. Thus, NSAIDs are a good choice for acute pain management after injury. NSAIDs are also used in the treatment of osteoarthritis in dogs due to their pain-killing and anti inflammatory properties.

As antipyretics, NSAIDs reduce body temperature in febrile states. PGE2, along with other factors is responsible for triggering the hypothalamus to increase body temperature during inflammation. NSAID inhibition of PGE2 activity in the hypothalamus may provide symptomatic relief (Dugowson and Gnanashanmugam, 2006).

NSAIDs inhibit platelet function because they prevent platelets from forming thromboxane TXA_2, a potent aggregating agent. Its inhibition causes prolonged bleeding.

1.3.1 Specific COX-2 inhibitors; the Coxibs

As cancer progresses, COX-2 participates in the arachidonic acid metabolism by synthesizing prostaglandins which can mediate various mechanisms related to cancer development such as: increase in angiogenesis, inhibition of apoptosis, suppression of the immune response, acquisition of greater invasion capacity and metastasis. Accordingly, overexpression of this enzyme in tumors has been associated with the most aggressive, poor prognosis cancer types, especially carcinomas. Therefore, treatments which use COX-2 inhibitors such as coxibs, whether administered as single agents or in combination with conventional antineoplastic chemotherapy, are an alternative for extending the survival of cancer patients (Nardi et al., 2011).

The recognition that the 2 isoforms of COX, despite being structurally similar have different behavioral functions largely because of the striking differences in their tissue expression and regulation, led pharmaceutical companies to develop selective COX-2–inhibiting NSAIDs referred to as coxibs, which reduced inflammation with a decreased propensity for GI complications. On the basis of observed upregulation of COX-2 in many cancers, the chemopreventive activity of coxibs was examined in animal models (Fischer et al., 2011).

In 1998, Robertson studied Ibuprofen-induced inhibition of cyclooxygenase isoform gene expression and regression of rat mammary carcinomas. Female Sprague–Dawley rats with 7,12-dimethylbenzathracene (DMBA)-induced mammary carcinomas were treated for 35 days with ibuprofen which resulted in significant reduction of tumour volume (P <0.05), and gene expression of both COX-1 and COX-2. The chemopreventive effect of a specific COX-2 inhibitor, celecoxib, against DMBA-induced mammary carcinogenesis in female Sprague–Dawley rats has also been investigated with dietary administration of celecoxib produced striking reductions in the incidence, multiplicity and volume of mammary tumours relative to the control group (Davies et al., 2002).

1.3.2 Traditional NSAIDs vs Coxibs; Risks; Adverse effects

Traditional non-steroidal anti-inflammatory drugs (NSAIDs) have been used to treat pain, but their long term use is limited due to serious gastrointestinal side effects. As the anti-inflammatory effects of NSAIDs were believed to be mediated by the inhibition of COX 2, and their gastrointestinal side effects by the inhibition of COX 1, researchers hypothesised that selective COX 2 inhibitors would provide a safer alternative to using traditional NSAIDs.

Cyclooxygenase (COX)-2 inhibitors have been developed with the goal of providing similar efficacy and greater safety compared with traditional nonsteroidal anti-inflammatory drugs (Fitzgerald, 2002). But, even though some studies have reported a lower incidence of upper gastrointestinal complications with selective COX 2 inhibitors than with traditional NSAIDs, recent concerns about the cardiovascular safety of selective COX 2 inhibitors have limited their use (Kearney et al., 2006).

Gastroduodenal ulceration is the best-characterized serious adverse event of NSAID therapy. It has also been speculated that use of daily dosed medications, may increase the risk for GI bleeding. NSAIDs injure the gut by depleting COX-1 derived prostaglandins which are the most important gastric cytoprotective agents and thereby cause topical injury to the mucosa (Clària, 2003).

Hepatic function, renal function, and age must be considered before prescribing and dosing. Aspirin can cause gastrointestinal upsets and ulcers in canines, just as in humans. It should be used with caution in small breed dogs as even a slightly high dose can be toxic.

There is an increase in renal dysfunction associated with longer-acting NSAIDs. Due to constitutive expression of COX-2 in the kidneys, there is an increase in renal toxicity when these agents are combined with antihypertensive agents and other potentially nephrotoxic drugs.

The selective COX-2 inhibitors do not inhibit platelet thromboxane A2, which is derived from COX-1. Studies depict that the COX-2 mediates prostacyclin suppression which increases the incidence of thrombogenic events, blood pressure and atherosclerosis. COX-2 inhibitors, in comparison with nonselective NSAIDs, alter the balance between antithrombotic and prothrombotic pathways in a way that promotes thrombogenesis. This is the scientific basis behind the emerging evidence of risk of cardiovascular events with use of COX-2 inhibitors.

Cardiovascular toxicity was seen clinically in studies demonstrating that the use of rofecoxib. Its use led to an increase in atherosclerotic events and finally the withdrawal of rofecoxib from the market. The evidence suggests that these drugs as a class increase the likelihood of a cardiovascular event, particularly in patients who are at increased risk (Dugowson & Gnanashanmugam, 2006).

2. MATERIALS AND METHODS

2.1 Animals and sampling

There were 42 dogs (40 females, 2 males) included in this study. All had mammary gland tumors. Their average age was 9.17 years (range, 4-15 years). Among these 28 had primary tumor at examination and 15 had relapsed neoplasm, because one dog had both, primary and relapsed tumuor. After the examination (routine blood work, two sided chest x-ray, abdominal ultrasonography), the dogs were operated for the primary and the relapsed tumours under general anaesthesia, by giving midazolam 0.5 mg/kg bw and fentanyl iv as initial bolus (loading dose) of 5 μg/kg, followed by a constant rate infusion of 6 μg/kg/hr, propofol 5mg/kg iv and isofluran 1.5-2.5 V/V %. Constant monitoring of blood pressure, ECG, oxygen pressure, and exhaled CO_2-referring to the arterial pCO_2 value was done after induction of anaesthesia. Affected mammary glands, or the whole chain of one sided mammary glands were excised along with the regional (axillar and/or inguinal) lymph nodes. Mean size of he tumours was 191,8 cm^3, which seems to be huge, but some animals had 10-16 cm tumours in diameter. Histopathology examination was made at the MATRIX Ltd.

After the surgical excision 31 dogs received non steroidal anti-inflammatory drug adiministration. Among all, 23 dogs received piroxicam at the rate of 0,3 mg/kg bw dose perorally, 18 dogs meloxicam 0.2 mg/kg bw initially, followed by 0.1 mg/kg bw dose twice a day for a day, and then 0.1 mg/kg bw dose continously. Twelve dogs were administred firocoxib at the rate of 0.5 mg/kg bw daily. These drugs were given for a minimum of four month period.

In all, 13 dogs received chemotherapy, inclusive of doxorubicin (n=7) in 30 mg/m2 dose iv. every 3 weeks, cyclophosphamide (n=1) in 150 mg/m2 dose iv. every 3 weeks, carboplatin (n=10) in 300 mg/m2 dose iv. every 3 weeks. Four dogs received combination protocol with different drugs.

2.2 Preparation of slides

For immunohistochemical analysis, samples from fresh tumors were surgically removed, fixed in 10% neutral formalin solution (for preserving cellular morphology), and then embedded in paraffin blocks. Serial sections were prepared from paraffin blocks, mounted

on silanized positively charged slides and then stored at 56 °C in a thermostat oven for 12 hours (for preventing peeling off on heat application). The processing of samples was carried out by the Introduction of the National Food Chain Safety Office, Veterinary Diagnostic Directorate, Mammalian Pathology Department.

2.3 The determination of Cox - 2 expression by immunohistochemical detection method

The determination of COX-2 expression by immunohistochemistry was done following Queiroga et al (2007), with minor modifications. The colleagues of the Veterinary Oncology and Hematology Center Ltd. (ÁHOK Kft.) lab performed a slight modification, on a recommendation from the National Cancer Institute, Hungary.

The sections were deparaffinized and rehydrated by sequential immersion in xylenes and a graded alcohol series. They were first immersed in xylene (Reanal Ltd.) for 2x20 –minutes, followed by soaking in absolute alcohol for 2x5 min, then for 2x5 min in 96% alcohol (Reanal Ltd.), and finally rinsed with distilled water.

The available 30% hydrogen peroxide (Reanal Ltd.) was treated with distilled water to the desired 3% concentration. Endogenous peroxidase was quenched by incubating the slides in 3% hydrogen peroxide for 10 minutes to block the endogenous peroxidase activity (in red blood cells, pseudoperoxidase). Then the slides were rinsed with PBS (phosphate buffered solution).

Heat-induced epitope retrieval technique was used for antigen retrieval from the specimens. This process was necessary because the previously used aldehyde-based fixative (formalin) causes protein cross-linking, resulting in the inability of some protein epitopes to bind complementary antibodies, and thereby giving weak staining for immunohistochemical detection of certain proteins. So the slide-mounted specimens were immersed in an alkaline, 1:3 dilution PBS Retrieve All in 1 solution (Signet Laboratories) pre-heated at 90 °C and left loosely covered for 10 minutes. Here the heat causes cross-linked protein epitopes to unfold, while the buffer solution aids in maintaining the conformation of the unfolded protein.

Following antigen retrieval, the slides were allowed to cool by washing in distilled water and subsequently in PBS for 5 minutes. The non-immune origin affinities were then blocked by of

4 % normal horse serum (Vector Laboratories) in 1:10 dilution with PBS for 20-minutes to prevent non-specific antibody binding. All types of antibody-epitope binding (specific and non specific) are governed by hydrophobic & ionic interactions, hydrogen bonding, and other intermolecular forces. This means that the same attractive forces can result in non-specific staining, i.e. binding of the primary antibody to amino acids other than those within the desired epitope of the antigen therefore causing an inability to visualize the antigen of interest in its appropriate cellular location. So, in order to increase specificity without impairing antibody-epitope binding, blocking agents are used prior to incubation of the sample with the primary antibody.

The slides were then treated for 2x5 min in PBS, and thereafter incubated with rat primary monoclonal IgG1 (rat, COX-2 aa. 368-604) antibody in 1:40 dilution at 4˚C overnight in a humidified chamber. Monoclonal antibodies are those that recognize a single epitope within an antigen and have low cross reactivity with non-specific antigens. They are typically produced from a single B cell of an immunized mouse, thereby generating an identical (clonal) population of antibodies all recognizing the same epitope of a specific antigen. The limited lifespan of a B cell can be overcome by fusing a specific antibody-producing B cell with a myeloma cell. The resulting B cell-myeloma hybridoma can provide a constant supply of highly specific monoclonal antibody.

On the following morning, the samples were removed from the humidity chamber. The humidity chamber should be used in every stage of staining in order to avoid drying of the tissue. Drying at any stage will lead to non-specific binding and ultimately high background staining. They were given washings in PBS for 2x10 minutes each. Following this, the sections were incubated with horse biotinylated antibody (Vectastain Elite ABC kit, Vector Laboratories) for 45 minutes. The purpose of treatment with the biotinylated antibody is to detect primary antibody binding by utilizing a labeled secondary antibody raised against the primary antibody host species. The indirect detection methods generally have a higher level of sensitivity and generate a more intense signal because of the potential for at least two labeled secondary antibodies to bind to each primary antibody.

Thereafter, the sections were rinsed in PBS for 2x5 minutes, and then incubated for 60 minutes in ABC solution. If a biotinylated secondary antibody is employed, the signal can be

significantly amplified by subsequent incubation with an avidin-biotin complex (ABC Method), or labeled streptavidin-biotin (LSAB Method). This process takes advantage of the strong affinity of avidin/streptavidin to bind biotin. Streptavidin is purified from the bacterium Streptomyces avidinii, is not glycosylated, binds four biotins per molecule and exhibits lower non-specific binding than avidin. Streptavidin may be conjugated to a detection enzyme (i.e. horseradish peroxidase or alkaline phosphatase) or fluorochrome.

Afterwards, the sections were rinsed for 2x5 minutes in PBS solution and then the antigen-antibody-peroxidase reaction was developed by incubation with a freshly prepared 3, 3'-diaminobenzidine (DAB) solution (Vector Laboratories) for 2 minutes. Visualization of the reaction takes place as the degraded substrate (H2O2) oxidizes the electron donor chromogen (DAB), forming an insoluble dark brown polymerized precipitate which basically indicates the location where the antibody sits on COX-2.

The final steps were as follows: the sections were successively rinsed twice with distilled water and counterstained with haematoxylin staining solution for 2 minutes and subsequently washed under running tap water for 2 minutes. Finally the specimens were dehydrated, mounted in DPX (DPX mountant, BDH Laboratory Supplies) and examined under microscope (Queiroga et al., 2007; Queiroga et al., 2010).

The implementation of immunohistochemistry is a sound inclusion of positive and negative controls. The positive control is meant to prove specific antibody binding. Our positive control was used in similarly healthy dog liver and kidney samples (Queiroga et al., 2007).

2.4 Evaluation of COX-2 Immunostaining.

COX-2 immunohistochemical staining was scored independently and in a blinded manner by two investigators (A. R. and A. S.) from 1728 tissue array cores, of which 152 (8.8%) either detached or did not contain tumor cells. The following scoring criteria of the tumor cells were agreed upon before the analysis: 0, no staining; 1 +, weak diffuse cytoplasmic staining (may contain stronger intensity in less than 10% of the cancer cells); 2 + , moderate to strong granular cytoplasmic staining in 10 –90% of the cancer cells; 3 + , over 90% of the tumor cells stained with strong intensity.

3. RESULTS

There were 13 benign (30,95%) and 29 malignant (69,05%) mammary tumours (Figure 5).

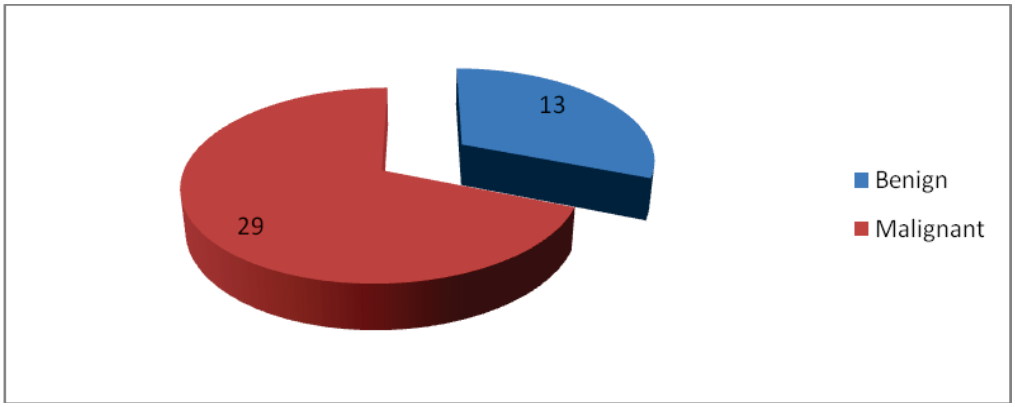


Figure 5. The number of benign and malignant mammary gland tumours

The histopathology examination revealed different tumour types (Figure 6).

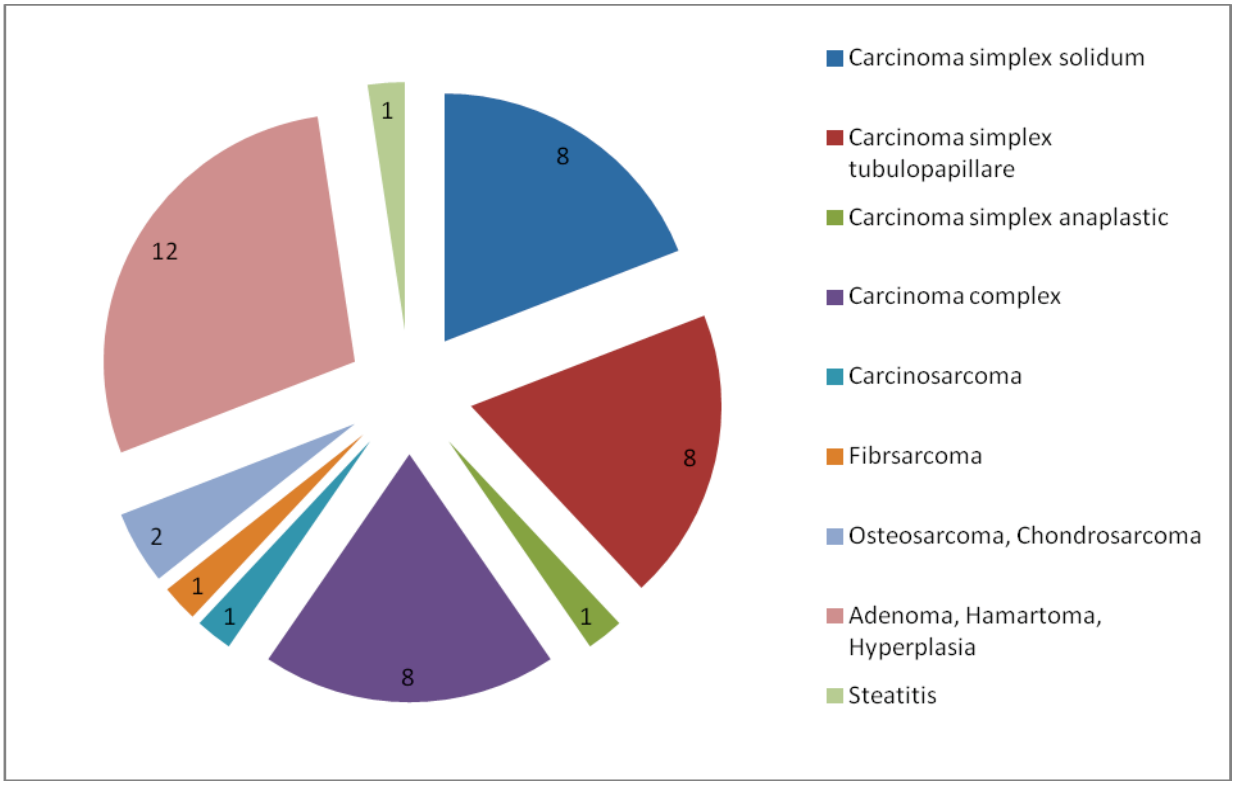


Figure x. Distribution of the histopathology results

The grading of the malignant tumours (n=29) were as follows; mostly of Grade I (48.27%), followed by those of Grade III (27.58%), and the least of Grade II (24.13%) (Figure 7).

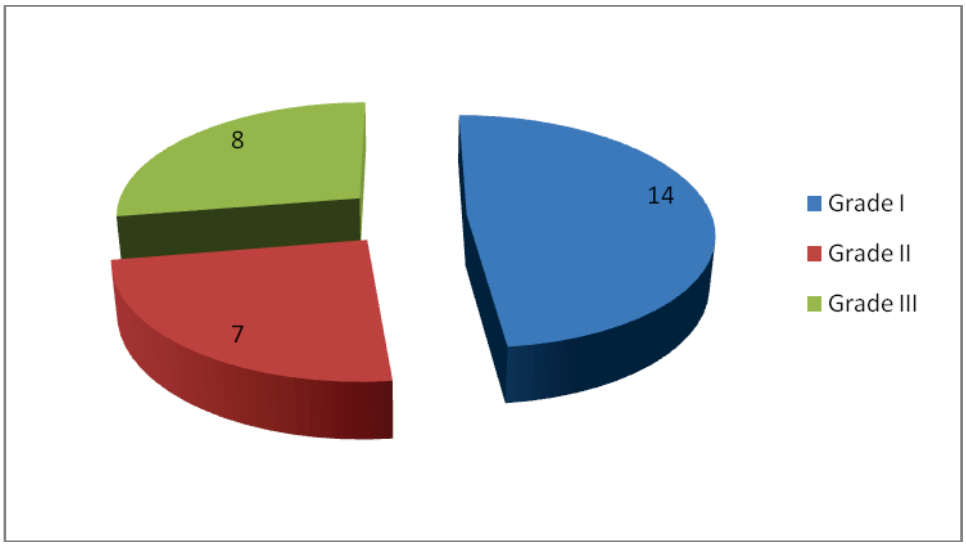
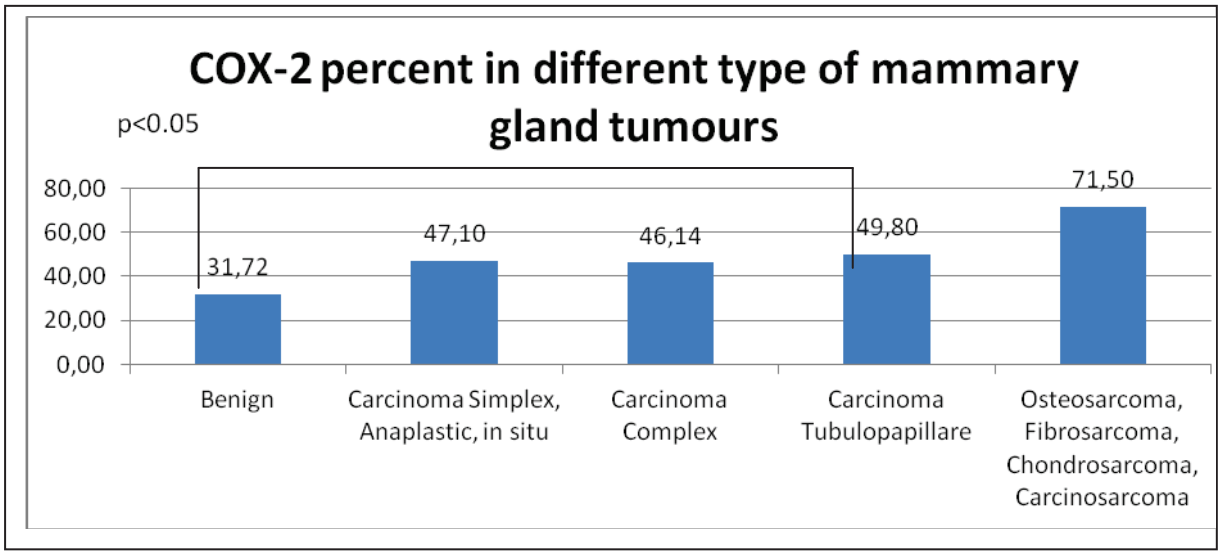


Figure 7. Distribution of grades in malignant mammary gland neoplasms.

Statistical methods

Analysis of Variance (ANOVA) and the Student's t-test was performed by SPSS 8.0 software and Microsoft Excel 7.0 programme. Pearson-correlation was performed by 'Free Statistics Software'(WESSA, 2013). The COX-2 immunohistopathological examination revealed the following results by the ANOVA test.

Fig 8. below shows COX-2 expression in different types of mammary gland tumours.



The COX-2 staining intensity was determined. The results were evaluated by the ANOVA test. The staining intesity showed significantly higher values in simple (anaplastic) carcinoma and the sarcoma types as compared with the benign tumours (Fig 9).

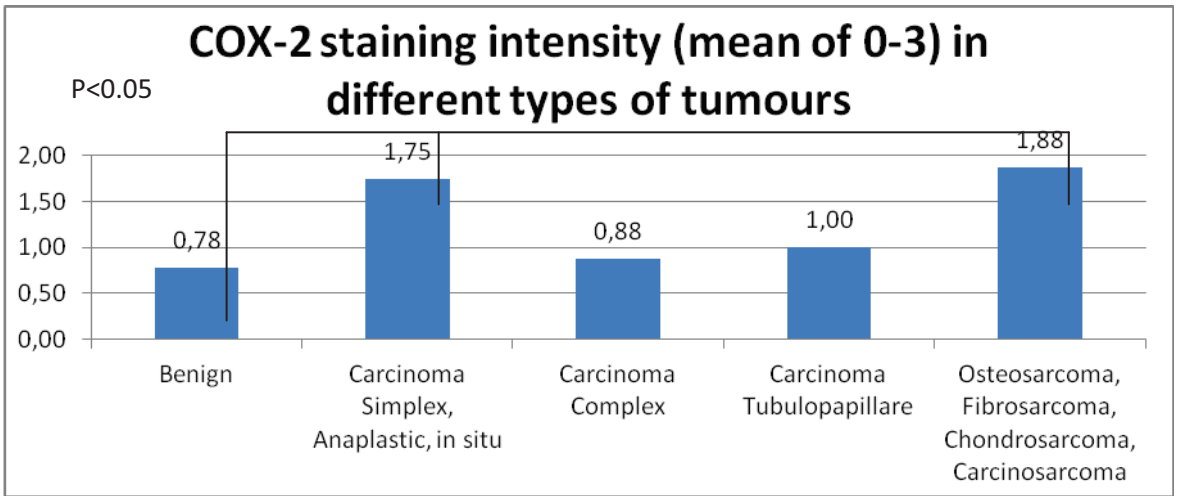


Fig 9. COX-2 intensity in different types of mammary gland tumours.

The survival of the animals with different types of tumours was significantly higher in benign tumours than in malignant ones (carcinoma complex and sarcoma types), and the relapse free period was also higher in benign lesions compared to the simple carcinomas and the sarcoma types (Fig 10). Test was performed by ANOVA.

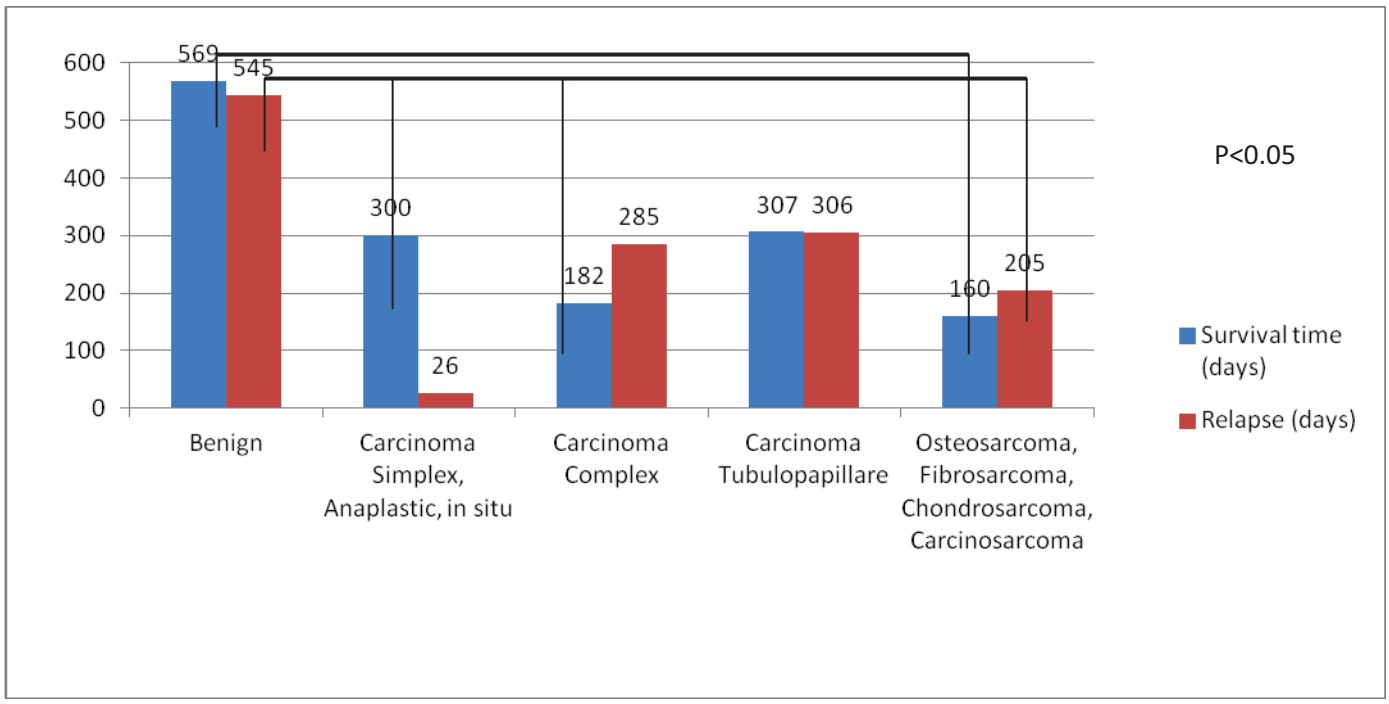


Fig 10. Survival and relapse free periods in different types of mammary gland tumours.

Those patients which had higher COX-2 percent than 50% showed marked lower survival time than those with COX-2 percent lower than 50% (Fig 11).

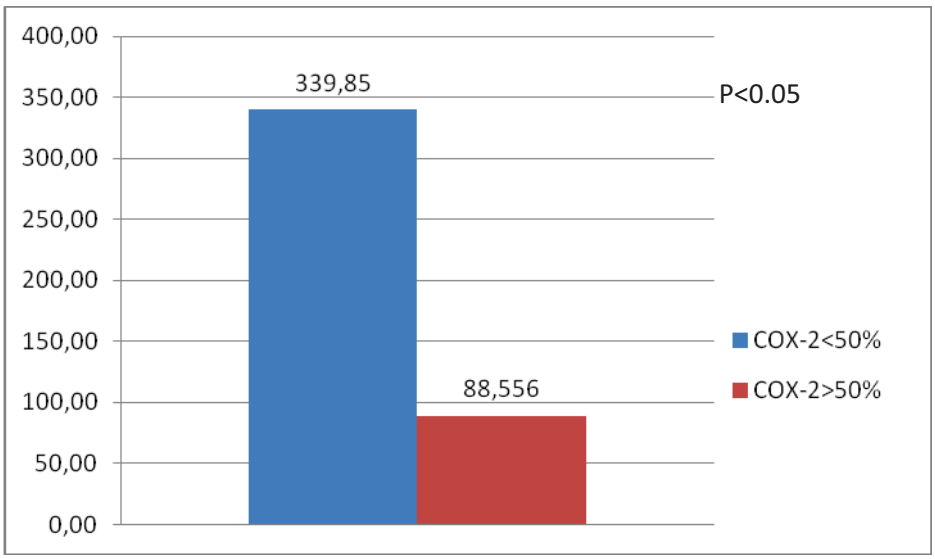


Fig. 11. Survival time of patients with lower and higher COX-2 percent than 50%.

Interestingly the patients with NSAID therapy did not show increase in survival time neither in malignant tumours, nor in carcinomas. Although, those patients lived significantly longer treated with NSAID therapy in which the tumour COX-2 percent was higher than 50% (Student's t-test).

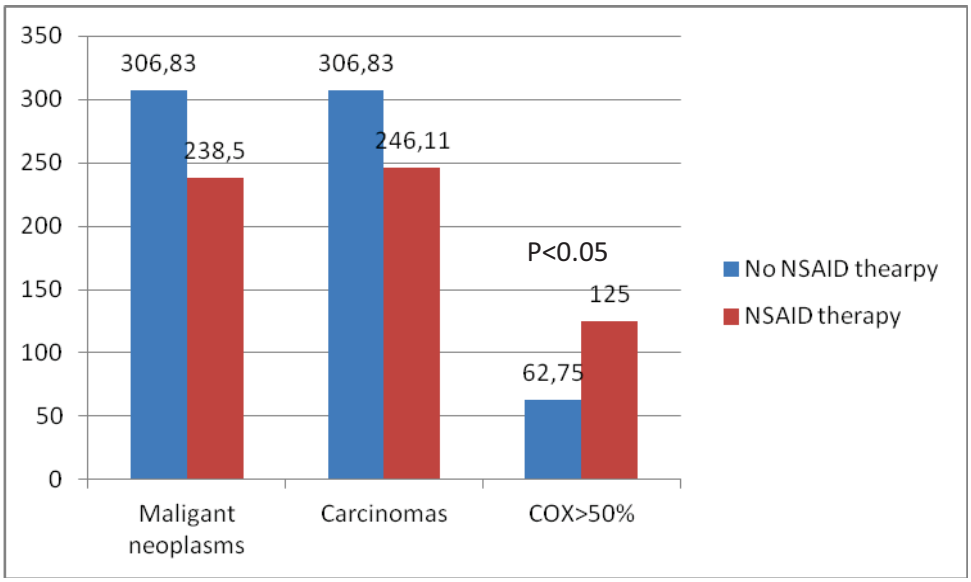


Fig 12. Survival and relapse free periods in different types of mammary gland tumours.

COX-2 expression of mammary gland tumours negatively correlated with the survival time and the relpase, but positively correlated with the tumour volume (Pearson correlation). (Table 1).

Table 1. Correlations in all patients with mammary gland neoplasias

Variables	Correlation coefficient (r-value)	P-value
COX-2 expression % : Survival time (days)	-0,50835998	<0.05
COX-2 expression % : Relapse (days)	-0,7817827	<0.01
COX-2 expression % : Tumour sizes (days)	0,455349141	<0.05

The use of different types of NSAIDs revelaed differences. Firocoxib alone correlated with the increased survival time (Pearson correlation) (Fig 13.)

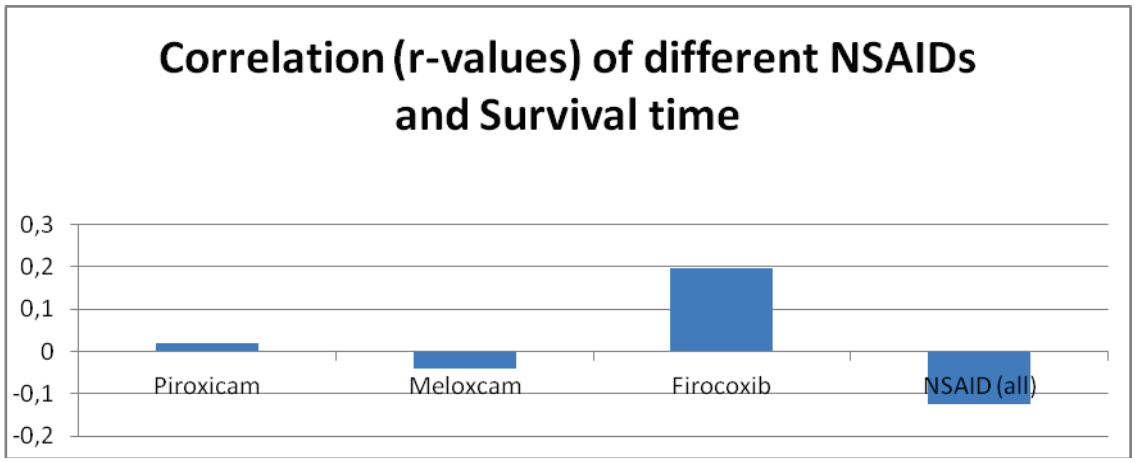


Fig 13. Correlation coefficients of the comparison of use of different types of NSAIDs and their survival time.

In case of different NSAID therapies, the survival time of the patients did not show increase, except in the case of firocoxib therapy. Those patients who recieved firocoxib showed significantly higher survival than those who did not receive NSAIDs, or received other NSAID than firocoxib (Student's t-test) (Fig 14.).

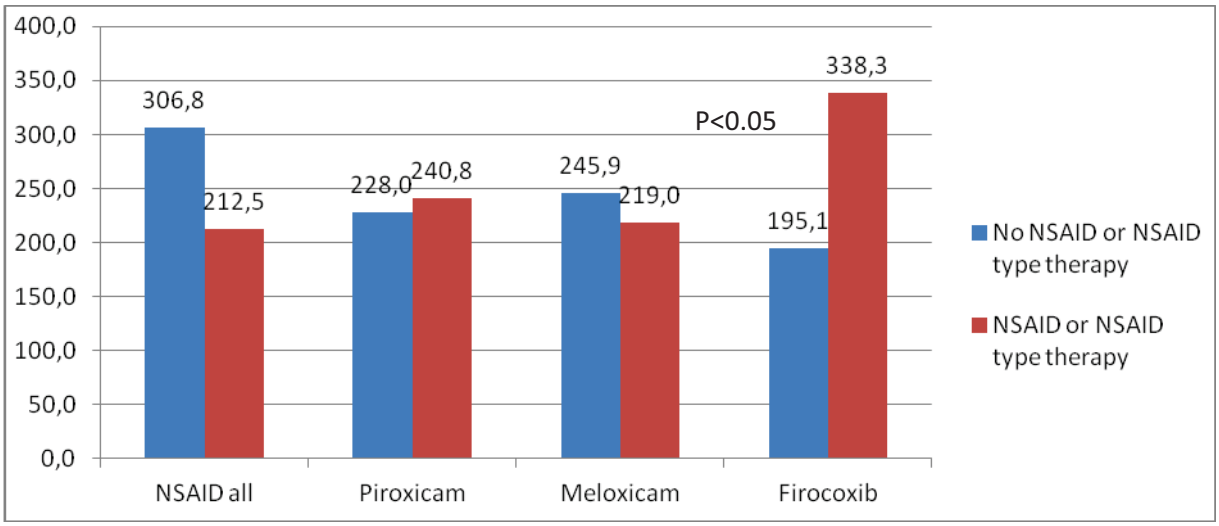


Fig.14. Survival times (days) with different NSAID types in therapy of malignant mammary gland tumours

4. DISCUSSION

Canine mammary tumors are of interest for both veterinary and human oncologists because they have epidemiologic, clinical, morphologic, and prognostic features similar to those of human breast cancer and are therefore suitable models with naturally occurring tumors (Rivera et al., 2009). Experimental studies in rats confirmed that the use of selective COX-2 inhibitor drugs exert a protective effect against tumor development in the gastrointestinal system, which verifies the importance of COX-2 in the carcinogenic process. Other studies found evidence of reduction in tumor growth and metastasis, which suggests an emerging role of selective COX-2 inhibitors in the prevention and treatment of cancer (Nardi et al., 2011)

Mammary gland tumors are the commonest tumors in female dogs, but rare in the male. In the present study, two males out of the total 42 dogs were affected with mammary neoplasia, which is significantly higher (4.7%) than that reported by others in previous studies. In 1999, Benjamin et al. documented that out of a total of 1,343 Beagles (including 671 males), only 2 males had mammary neoplasia (less than 0.5%). This also fell in line with most observations that suggest about 1% incidence of mammary tumors in male dogs as compared to the females.

In this study, the malignant tumors accounted for 69.05 % of the total mammary tumors, which is higher than the values usually reported in literature as the incidence of malignant tumors stated in most studies has been around 50% (Misdorp, 2002, Doré et al., 2003; Lavalle et al., 2009). However, Hellmén et al. (1993), recorded the incidence of malignant tumor to be 68%, which is consistent with our findings. This can be due to the late presentation of the affected animals. Similarly, Bauman et al. (2004) also report 71% mammary malignant tumours in a group of 136 females. The higher incidence of canine mammary malignancy in the present study as well as in studies by some others, might be due to the late presentation of the affected animals. Often the earlier stages go unnoticed or ignored by the owner, thus causing a higher incidence of malignant tumors than benign ones due to progression of neoplasia.

Mammary neoplasia has been reported in both mixed breeds and purebred dogs affecting large and small breed dogs alike. The breeds reported to have an increased incidence of

mammary neoplasia, include dachshunds, cocker spaniels, toy poodles, german shepherds, besides mixed breed dogs (Tavasoly et al., 2013). In this study, it was found that the mixed breed dogs (13) had the maximum occurrence of tumors. However, some pure breeds affected were shnauzer, spitz, bolognese, shih tsu, chihuahua, west highland white terrier, american staffordshire terrier, beagle, rottweiler and boxer. An absence of significant correlations with breeds in this study may have resulted due to relatively small size of the data set, not suitable for deriving such conclusions.

A similarity between human breast tumors and canine mammary tumors is found regarding incidence, behavior and histological origin. The appearance of mammary tumors in female canines corresponds to the development of these tumors in women. The risk of developing both these tumors increases starting with the age of 6 years in bitches, which is approximately equivalent to 40 years in women (Brunelle et al., 2006; Baba and Câtoi, 2007). The average age of incidence was 9.17 years in this study, which is consistent with literature. Middle-aged female dogs are primarily affected, the mean age being 8 to 11 years (Zatloukal et al., 2005; Sorenmo et al., 2011). It is uncommon to have mammary gland tumors below two years of age (Alenza et al., 2000) and this was confirmed in this study as we found the lowest age affected to be four years.

From the 29 malignant tumors examined in the present study, 14 (48.27%) had well-differentiated (grade I), 7 (24.13%) had moderately differentiated (grade II) and 8 (27.58%) had poorly differentiated (grade III) tumors. The percent grading of mammary neoplasms in this study is not in consonance with other researchers, who reported varying results in their respective studies (Karayannopoulou et al., 2005; Shafiee et al., 2013). In a study by Karayannopoulou, 85 cases were examined, out of which 27 (31.8%) had well-differentiated, 28 (32.9%) had moderately differentiated and 30 (35.3%) had poorly differentiated carcinomas. Shafiee et al. (2013) examined 15 cases, out of which, 2 (13.3%) had grade I, 6 (40%) grade II and 7 (46.7%) grade III tumours.

COX-2 immunoexpression is known to be higher in malignant tumours than in their benign counterparts (Doré et al., 2003; Queiroga et al., 2007). The results of the evaluation of COX-2 staining intensity on a scale of 0-3 showed significantly higher values in simple (anaplastic) carcinoma (1.75) and the sarcoma (1.88) sub-types as compared to the benign tumours

(0.78). The study by Doré et al. in 2003, revealed that the incidence of COX-2 expression and the intensity of the COX-2 signal were higher in adenocarcinomas than in adenomas ($P < 0.001$). While, normal mammary gland tissues did not express COX-2, some adenomas (24%) displayed COX-2 expression. In these tumors, COX-2 appeared as a diffuse pale cytoplasmic staining. In contrast, they found that malignant tumors expressed COX-2 much more than benign tumors as well as the intensity of COX-2 expression was significantly higher in malignant tumors. Similarly, Queiroga et al. (2007), showed that malignant tumours had higher values of COX-2 expression, and COX-2 staining was particularly intense in histological types classically associated with high malignancy. In a study of 84 canine malignant mammary tumors, Brunelle et al. (2006) found that approximately half of the tumors were COX-2 positive. Similarly, Heller et al. (2005) found that 46% of the 37 adenocarcinomas were COX-2 positive. However in humans, the reported incidence of COX-2 expression in breast cancer tissues varies greatly, ranging from 5 to 100%.

The current study depicted that the survival of the animals was significantly higher in benign tumors than in malignant ones (carcinoma complex and sarcoma sub-types). The prognosis for patients with benign tumors is deemed favourable (Misdorp et al. 1999; Philibert et al. 2003; Lorenzová et al., 2010). In a study by Lorenzová, causes of death and survival periods of females after the surgical excision of mammary tumour and their relation to the tumor histological sub-type was evaluated. In this study of 39 female dogs, none of them died as a result of the tumor in benign mammary neoplasia, while in the patient group with malignant tumors, 25% died, thus showing a worse prognosis for malignant canine mammary tumors.In addition to this, there was more time before relapse in benign lesions as compared to the malignant tumors. We found that the simple carcinoma, anaplastic carcinoma and *in situ* group had the fastest relapse rate that is merely 26 days after excision. This could be due to the invasive nature of the tumor leading thereby to incomplete excision. Morris et al. (1998) reported in their survival evaluation study that 60% females died after surgery of invasive carcinoma sub-types and 24% females died within 2 years after surgery of non-invasive carcinomas due to local relapses, metastases in the lungs or both of these. In agreement to this, we found an inverse relation between the COX-2 expression and the time before relapse ($p<0.01$). This means that higher the expression of COX-2, the faster it is for the tumor to re-occur.

In the present study, the patients who had higher COX-2 percent than 50%, showed a markedly lower survival time than those with COX-2 percent lower than 50%. In human breast cancer and canine mammary tumor studies, overexpression of COX-2 has been linked to poor prognosis by some researchers (Ristimäki et al., 2002; Queiroga et al., 2005). In female dogs, Doré et al. observed higher COX-2 expression in malignant than in benign tumors. Elevated COX2 expression is associated with reduced survival in many types of cancer. This appears to be related to the induction of angiogenesis and proliferation. A study aimed to evaluate COX-2 expression and microvessel density in canine mammary carcinomas and to correlate them with overall survival of the animal demonstrated that increased microvessel density and increased COX-2 expression were linearly related in the malignant canine mammary neoplasia with worse prognosis and shorter overall survival (Lavalle et al., 2009). Similarly, the current study also found a negative correlation between COX-2 expression and patient survival ($p<0.05$).

The effect of NSAIDs on chemoprevention and tumor regression has been shown in animal models, epidemiologic studies, and in the treatment of patients. The exact biochemical and cellular mechanisms underlying each of these phenomena is only partially understood (Moore et al., 2000). It was interesting to note that the patients who received NSAID therapy in this study did not respond positively to it, neither in malignant tumours as a whole group, nor in the carcinomas. Instead there was a decrease in the survival time of these patients after receiving NSAID therapy. However, it was noted that in cases where the COX-2 expression was higher than 50%, treatment with NSAID therapy yeilded positive results prolonging the life of the patients. Queiroga's results in the study conducted in 2005, show that COX-2 concentrations were significantly higher in the Inflammatory mammary carcinoma (IMC) group with respect to the non-IMC malignant tumors, suggesting a special role for COX-2 in the inflammatory type of cancer. On these lines, from our results it could be inferred that the tumors that express COX-2 at a higher rate are generally more related to inflammation, and therefore they are more responsive to anti-inflammatory drug therapy.

We found a positive Pearson correlation between tumor size and COX-2 expression. It has been reported that this correlation exists also in studies concerning breast cancer in humans (Arun and Goss, 2004). Dogs with mammary tumours less than 3 cm in diameter have a significantly better prognosis than dogs with larger tumors. In a study by Chang et al., dogs

with tumors larger than 5 cm in diameter were more likely to develop metastases than dogs with smaller tumors and were 7 times more likely to die in the first 2 years after surgery (Chang et al., 2005).

In a study by León-Artozqui and Morcate (2008), an improvement in quality of life in patients with transitional cell carcinoma of the bladder after treatment with firocoxib was observed and there was a significant increase in patient survival. In another study with canine mammary tumors, the firocoxib clinical trial included 31 dogs. Results were varied, however, there was an overall positive response both prior to surgery and as a means to control metastasis (Castillo, 2008). Corresponding to these results we found a significant increase in the survival index of patients in our study, who received firocoxib instead of any other drug administered (meloxicam, piroxicam, all three NSAIDs).

SUMMARY AND CONCLUSION

Canine mammary neoplasia is considered as an excellent naturally occurring animal tumor model for human breast cancer. Nearly half or more of the canine mammary neoplasms are malignant, overexpress COX-2 and show poor prognosis, particularly those having inflammation. Therefore, the use of anti-inflammatory drugs such as NSAIDs has recently been considered useful in prolonging the life span and in palliative care of human mammary cancer patients. However, there seems to be scarcity of information on the role of NSAIDS in canine mammary tumor.

The present study was conducted with a view to evaluate COX-2 expression in canine mammary tumor and its relation to NSAID therapy. For this purpose, 42 dogs (40 females, 2 males) were included in this study with an average age of affected dogs 9.17 years (range, 4-15 years). Among these 28 had primary tumor at examination and 15 had relapsed neoplasm. (One dog had both primary and relapsed tumor). COX-2 immunohistochemical staining was scored independently and in a blinded manner by two investigators from 1728 tissue array cores stained by LSAB method. The histopathology revealed that there were 13 benign (30,95%) and 29 malignant (69,05%) mammary tumours. Out of the latter, 15 were simple carcinomas, 8 complex carcinomas, 1 carcinosarcoma and the rest sarcomas. When graded 14, 7 and 8 belonged to the grade 1, 2 and 3 respectively.

The COX-2 expression was elevated, in general, in malignant mammary tumors as compared to benign counterparts and more so in sarcomas and the staining intensity was significantly higher in both carcinoma and the sarcoma sub-types as compared to the benign tumors. The survival of the animals with different types of tumors was significantly higher in benign tumors than in malignant ones (carcinoma complex and sarcoma sub-types), and the relapse free period was also higher in benign lesions compared to the simple carcinomas and the sarcomas. Those patients which had higher COX-2 percent than 50%, showed markedly lower survival time than those with COX-2 percent lower than 50%. The patients with NSAID therapy did not show an increase in survival time neither in malignant tumours, nor in carcinomas although, the patients in which the tumour COX-2 percent was higher than 50% lived significantly longer when treated with NSAID therapy.

In a nutshell, COX-2 expression of mammary gland tumors negatively correlated with the survival time and the relapse, but positively correlated with the tumor volume. The patients who recieved firocoxib showed significantly higher survival than those who did not receive NSAIDs, or received other NSAID than firocoxib viz.piroxicam and meloxicam.

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ACKNOWLEDGEMENT

This thesis was my first real experience in scientific writing. It was difficult at times, and enjoyable at others. But, it was definitely not my hardwork alone. A lot of people and their experiences helped me throughout this work that I cannot go without thanking.

First and foremost I am thankful to my alma mater, Veterinary Faculty of Szent Istvan University, Budapest, Hungary for providing the opportunity and platform to carry out this research work. I am grateful to Dr. Vörös, Károly, Head of the Department of Internal Medicine for allowing me to do the thesis my favourite department. I would like to say a big thank you to my supervisor, Dr. Péter Vajdovich without whom this work would have been impossible. All my gratitude goes to him, for giving me the chance to participate in this work and providing to me each and every information that I needed. I am highly obliged to him, for taking out time from his busy schedule, for evaluating the results of the experimental data and correcting this study. Any words will be insufficient to express my heartfelt gratitude towards him.

Next I would like this opportunity to thank my benefactors, the FAO, and the Ministry of Agriculture, Hungary for providing me a full time scholarship to complete my education in this prestigious university. Without their monetary support I wouldn't be able to sustain myself here for 5 years away from home. I also thank Mr. Kiss, László, Head of the Marek Joszef Collegium, for providing accommodation during all these years including the period of this study.

I thank my beautiful family from the bottom of my heart. I was extremely lucky to have a guide in the form of my father, Dr. Naresh Sood, who helped me at every step whenever I needed him. I truly thank him, wishing that someday I could know half as much as he does. I am thankful every single day to my mother, Mrs. Ranjana Sood, who can understand me so well. In her, I find the best support and comfort that a child could have. It was her faith that I would finish in time, which kept me going. Special thanks to my brother, Kushal Sood for cheering me up everytime I felt low.

I wish to thank my friends both in India and Budapest, especially Kanika Kapoor, Christina Thrasivoulou and Barbara Köglberger for their constant encouragement and advice.

I dedicate this work to the loveliest grandparents who are still blessing me from above, to Biji and Pitaji.

"Thought is the wind, knowledge the sail, and mankind the vessel" - Augustus Hare

Thank you all. Ruhi Sood

LIST OF ABBREVIATIONS

AA: Arachidonic acid

ANOVA: Analysis of Variance

Bcl-2: B-cell lymphoma 2

CD31(PECAM-1): Platelet Endothelial cell Adhesion Molecule-1

COX : Cyclo-oxygenase

CT: Computed tomography

DAB: diaminobenzidine

DMBA: Dimethylbenzathracene

E-cadherin: Epithelial cadherin

EGF: Epidermal growth factor

ER: estrogen receptor

FNAB: Fine needle aspiration biopsy

GI: Gastro intestinal

H2O2: Hydrogen peroxide

HER-2: Human epidermal growth factor receptor 2

Hsp70: Heat shock protiens 70

Hsp90: Heat shock proteins 90

IgG1: Immunoglobulin G1

LSAB: Labeled Streptavidin Biotin

Mcl-1: Myeloid cell leukemia 1

MIB-1: Mindbomb 1 gene

NSAID: Non steroidal anti-inflammatory

p53: protein 53

PBS: Phosphate buffered saline

PG: Prostaglandin

PGE2: Prostaglandin E2

PGG2: Prostaglandin G2

PGH2: Prostaglandin H2

P-gp: P-glycoprotien

PR: Progesterone receptor

RIE: Rat intestinal epithelial

tNSAIDs: traditional Non steroidal anti-inflammatory drugs

TXA_2 : Thromboxane

VEGF: Vascular endothelial growth factor

β-catenin: Beta-catenin

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Title of document (to upload): E**valuation of COX-2 expression in canine mammary tumors and its relation to NSAID therapy.**

Publication data of document: 2013

Number of files submitted: 1

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