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*Research article*

# TUMOR MARKERS IN DOGS WITH MAMMARY GLAND TUMORS

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The aim of this study was to determine and compare values of carcinoembryonic antigen (CEA) and cancer antigen (CA 15-3) in 50 bitches with mammary tumors and 150 clinically healthy dogs. A modified procedure was used to determine the CEA and CA 15-3 markers with the human kits using the radioimmunoassay method (RIA). Samples collected from extirpated tumors of mammary glands were histologically processed and classified as per WHO guidelines. The mean values of the carcinoembryonic antigen markers ± SD were as follows: control group 0.89 ± 0.79, group with mammary gland tumor 1.53 ± 1.15. The values of cancer antigen markers CA 15-3 ± SD were: 1.52 ± 0.66 and 2.87 ± 1.11, respectively. The statistical significance for the carcinoembryonic antigen marker between groups was $P < 0.0001$. The cancer antigen CA 15-3 values between groups were also statistically significant with $P < 0.0001$. The results of the present study show that there are significant differences in both antigens between the control group and groups with mammary gland tumor in dogs.

**Keywords:** dog, tumor markers, CEA, CA 15-3, mammary gland tumors

## INTRODUCTION

Spontaneous malignant tumors in dogs closely recapitulate their human counterparts with respect to clinical presentation, histological features, molecular profiles, and response and resistance to therapy, as well as the evolution of drug-resistant metastases [1].

However, there are some differences between mammary tumors in dogs and breast cancer in humans, including the prevalence in dogs, which is about three times higher than in women [2]. Both dogs and people benefit from this approach, as it provides dogs with access to cutting-edge cancer treatments and helps ensure that people are given treatments which are more likely to succeed [1]. From this perspective the

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dog represents an optimal model organism to study cancer biology in a comparative setting, as many genes exhibit a high degree of homology to their human counterparts [3]. Mammary tumors are a common malignancy in female dogs, with 50% being malignant and half of them having metastasized at the time of diagnosis [4]. In bitches, mammary gland tumors are malignant or benign, and originate from different types of breast tissue (epithelial or glandular tissue, mesenchymal or interstitial tissue). The majority of these tumors are classified as epithelial tumors - carcinomas. Approximately 65% of all mammary gland tumors are observed in the caudal pair. Risk factors for tumor formation include exogenous sex hormones, repeated pseudopregnancy, and mastopathy [5]. The incidence of mammary gland tumors is higher in intact bitches. Although modern technology and radiological screening procedures exist, more practical and sensitive laboratory methods, which can help to detect various neoplasia and provide quantitative assessment regarding growth, invasiveness, metastasis, and therapy are desirable [6]. In veterinary medicine, until now, initial clinical examination – adspection and palpation of lesion, TNM classification of tumors, followed by fine-needle aspiration cytology which confirmed or disproved malignancy were used for the evaluation of mammary gland tumors. Before surgery, biochemical and hematological examinations are performed. After surgical extirpation, the type of tumor is identified histologically according to the WHO guidelines [7]. Tumor cells display protein molecules on their surfaces known as tumor-associated antigens, which may be present in higher concentrations than usual in other tissues, serum, urine, or body fluids of patients with cancer [8]. Thus, the elevation of these markers can be helpful in early diagnosis, determining prognosis, following a course of treatment, predicting the response or resistance to specific therapies and surveillance after primary surgery [9]. Their concentration in the blood serum and plasma is determined by various immunochemical methods including RIA (radioimmunological methods). Only small amounts of tumor markers remain in the tumor tissue where they can be detected by immunohistochemical methods or their concentration in tissue cytosols can be measured [9]. The parameters of basic tumor markers are in human medicine presented as CEA (carcinoembryonic antigen) and CA 15-3 (carbohydrate antigen). CEA was one of the first tumor markers identified and described [10]. Some studies suggest that positive CEA values found in the serum at the time when a primary breast tumor is diagnosed represent a negative prognostic factor. Carbohydrate antigen CA 15–3, the product of the mucin 1 gene (MUC1), is a large transmembrane glycosylated molecule aberrantly overexpressed in many adenocarcinomas in an underglycosylated form and then shed into the circulation. High values of CA 15-3 can be connected with severely affected tissue and a poor prognosis [11]. Molina et al. (2003) [12] suggested that women with a high concentration of CA 15-3 in the blood serum have a worse prognosis, and showed that the CA 15-3 antigen can be the first marker for relapses, as well a reliable prognostic indicator of breast tumor patients. In veterinary medicine, many human diagnostic procedures are used, such as biochemical and hematological

tests, or determination of hormone concentrations using human kits. In human medicine, for the determination of breast tumors, the basic markers CEA and CA 15-3 are used. In veterinary medicine, there is information about the determination of the tumor markers CEA and CA 15-3 by use of RIA methods from our preliminary study [13].

The aim of the study was to establish values of the tumor markers CEA and CA 15-3 in clinically healthy dogs and bitches with mammary tumors.

## MATERIAL AND METHODS

### Animals

The group of mammary gland tumors included 50 bitches (aged 5 to 15 years). The group of animals examined consisted of 150 clinically healthy dogs without evident clinical changes in the mammary gland (aged 8 months to 12 years). Most dogs in our study were mixed breed. Specific patient data included signalment, medical history data and description of the current health state. Data on animal breeds can be found in Table 1 and Table 2.

**Table 1.** Control group

| Breed of dog | Number of dogs |
|---|---|
| Mongrel | 80 |
| German Shepherd | 22 |
| Mongrel x German Shepherd | 18 |
| Czechoslovak Shepherd | 4 |
| Yorkshire terrier | 4 |
| Dalmatian dog | 4 |
| Rottweiler | 3 |
| Cocker Spaniel | 3 |
| Labrador Retriever | 3 |
| Poodle | 2 |
| Schnauzer | 2 |
| Border collie | 1 |
| Fox-terrier | 1 |
| Aberdeen terrier | 1 |
| Jack Russel terrier | 1 |
| Thailand ridgeback | 1 |

**Table 2.** Group with mammary gland tumours

| Breed of dog | Number of dogs |
|---|---|
| Mongrel | 16 |
| German Shepherd | 11 |
| Cocker Spaniel | 6 |
| American Staffordshire terrier | 3 |
| Poodle | 2 |
| Yorkshire terrier | 2 |
| Miniature dachshund | 2 |
| Irish setter | 2 |
| Springer Spaniel | 2 |
| Sleuthhound | 2 |
| Border collie | 1 |
| Akita Inu | 1 |

## Clinical examination

All animals were subjected to an initial clinical examination, i.e. visual inspection and palpation of all sets of mammary glands, regional lymph nodes and possible formations. If mammary gland pathology was diagnosed as a malignant tumor, cancer stage was determined using the TNM system [14]. Subsequently, three thoracic X-ray projections were performed: dorsoventral or ventrodorsal; and right and left laterolateral projections. Before surgery, animals underwent biochemical (BCH) and hematological (H) examinations as well as preoperative ECG. Venous blood was collected from *v. cephalica antebrachii* or *v. saphena medialis (lateralis)* of all bitches. Blood samples were placed in the test tubes with agglutinative gel using sterile single-use needles (21G x 38 mm, 0.8 x 38 mm). The blood was then centrifuged at 1,372 × g for 10 min (Eppendorf centrifuge 5702, max RCF 3,000 × g) and the blood serum was separated. The remaining blood serum was stored at -18 °C until the tumor markers were determined. Transport of blood sera to the laboratory was carried out in a Pharmacy Refrigerator Portable Vaccine Cooler Box where the temperature was kept constant (2–8 °C) until arrival.

## Surgical removal

A variety of procedures for removing tumors on the mammary gland, and choice of procedure were determined by size, fixation to surrounding tissue and number of lesions. Surgical removal was performed as per surgical oncological protocol designed by Gilson and Stone (1990) [15]. After surgical removal, the type of tumor was established by histological methods.

### Histological examination

In each animal, a sample (1 $cm^3$) was collected from the margins between healthy and diseased tissue of the extirpated neoplasm and fixed in 10% neutral formalin. After 24 hours, the sample was embedded in paraffin and cut into 4um slices, which were then stained with hematoxylin-eosin. All slides were examined under a light microscope (Microscope RED-223 LED, Motic, Camera Moticam 3+, 3.0 MP, Xiamen, China) at × 40 and × 100 magnifications. Mammary gland tumors were classified according to WHO guidelines [7].

### Determination of tumor markers CEA and CA 15-3

Tumor markers were determined using a radioindicative method – immunoradiometric analysis (IRMA) with the use of commercially available kits designed for human medicine. Both determinations (CEA and CA 15-3) are based on the sandwich method using two monoclonal antibodies against two different epitopes, which work independently of each other. These markers are not currently available in veterinary medicine. Therefore, a comparison of reagent composition, commercial kits, and commonly used human and veterinary diagnostic sets were performed (Catalog of diagnostic sets). Upon comparison, minimal variations were detected during the incubation period, in the dilution of samples and pipetted volumes, and therefore it was necessary to optimize the reagents and steps for determination to accurately measure the concentrations of the markers from calibration curves. For the determination of CEA markers, we used kits from Beckman-Coulter, Inc. (Prague), and for the determination of CA 15-3 markers, we used kits from Izotop (Izotop, Hungary). The obtained data were calculated on machines from Backman Coulter-Immunotech-LB 2111 (multicrystal gamma counter), with software from LBIS (Backman Coulter, Bratislava, Slovak Republic). Values of CEA markers were measured in ng/ml and values of CA 15-3 markers in IU/ml. In human medicine, the standard values for CEA markers are up to 5 ng/ml and for CA 15-3 markers, the norm is 30 IU/ml with an upper limit of 35 IU/ml. A reference value usually describes the variations of a measurement or value in healthy individuals. Cut off value serves as a marker that is located under most of the values of healthy patients and patients with benign diseases. We determined the specificity of both tests to be 95%. The laboratory was under external quality assessment by SEKK-Pardubice (Czech Republic) for these markers since year 2005.

### Modified determination of CEA markers

Prior to determination, all reagents were brought to laboratory temperature and thoroughly mixed. The contents of laboratory bottles containing lyophilized reagents, apart from the samples, were diluted in redistilled water whose volume was marked by labels. The cleaning solution was prepared by dilution with 950 ml of redistilled water.

### Determination procedure

In antibody-coated test tubes, 10 µl of sample and 300 µl of a radioindicator were mixed. After 2 hours of incubation at laboratory temperature, and constant mixing (at >280 vibrations/min), the contents were carefully removed and flushed twice with 2 ml of cleaning solution. The binding activity was measured using a gamma counter over the course of 2 minutes. The calibration curve intervals for measurements ranged from 0.50 to 325.0 ng/ml. The margin of error for the diagnostic kits, as stated by the manufacturer, was 0.20 ng/ml.

### Modified determination of CA 15-3 markers

As in the determination of markers, all reagents for the determination of CA 15-3 markers were brought to laboratory temperature and thoroughly mixed. Lyophilized reagents were reconstituted with redistilled water according to the manufacturer's guidelines. We used 1:1 dilution of dilution solution and distilled water. The samples were diluted at a ratio of 5 µl of serum to 500 µl of diluted dilution solution.

### Determination procedure

In antibody-coated test tubes, 10 µl of diluted sample and 200 µl of antiserum were mixed. After 1 hour of incubation at laboratory temperature and constant mixing (at > 280 vibrations/min) the contents were carefully removed and triple-flushed with 2 ml of cleaning solution. Then, 200 µl of radioindicator was added to each reagent tube. After 1 hour of incubation, the contents were carefully removed and triple-flushed with 2 ml of cleaning solution. Binding activity was measured using a gamma counter over the course of 2 minutes. The calibration curve intervals for measurement ranged from 6.25 to 300.0 IU/ml. The margin of error for the diagnostic kits, as stated by the manufacturer, was 2.0 IU/ml.

### Statistical analysis

Unpaired t-test was used to compare CEA and CA 15-3 values in control dogs compared to the bitches with mammary gland tumor.

### The ethical approval process

We confirm that the owners of patients in this study and the head of Small Animal Clinic approved the medical process. Each owner's consent was obtained before the collection of samples. The study was conducted according to the regulations of the local Institutional Animal Care and Use Committee.

## RESULTS

Tumor markers CEA and CA 15-3 were determined in 50 bitches with mammary gland tumors and 150 clinically healthy bitches.

### Surgical removal

In the 50 observed bitches, the following procedures were performed: mamectomy (n=9), regional mastectomy – 3, 4, 5 mammary gland (n=21), regional mastectomy – 1, 2 mammary glands (n=6), unilateral mastectomy (n=13) and bilateral mastectomy (n=1).

### Histological finding

Histology confirmed malignant mammary gland tumors, including solid carcinomas (n=12), complex carcinomas (n=10), carcinomas with squamous differentiation (n=9), adenocarcinomas (n=7), cystadenocarcinomas (n=6), spinocelullar carcinoma (n=3), mixed malignant tumors (n=2), and scirrhous carcinoma (n=1).

### TNM system

In the present study, all tumors were malignant (T 1-3). These tumors were classified by histological examination after surgical removal. TNM classification of all 50 neoplasms was as follows: T1 (7/50), T2 (25/50) and T3 (18/50). T and N stages were determined by clinical examination of tumor and lymph node size. Lymph nodes (N) were enlarged in 15 bitches. Cytological or histological examinations of lymph nodes were not performed. No visible metastases (M) were noted in the lungs by X-Ray examination of any of the 50 bitches before the surgery.

### Breed distribution and age

Breeds with high incidence of mammary gland tumors (Table 2) included mongrels (n=16), German Shepherd (n=11), Cocker Spaniel (n=6), American Staffordshire terrier (n=3), Poodle, Yorkshire terrier, Miniature dachshund, Irish setter, Springer Spaniel, Slethhound (each n=2), Border collie, Akita Inu (each n=1).

50 bitches with mammary gland tumors were aged from 5 to 15 years. Among 150 clinically healthy dogs without evident clinical changes in the mammary gland, the age ranged from 8 months to 12 years.

Breeds of clinically healthy bitches (Table 1) included Mongrel (n=80), German Shepherd (n=22), MongrelxGerman Shepherd (n=18), Czechoslovak shepherd, Dalmatian dog, Yorkshire terrier (each n=4), Labrador Retriever, Rottweiler, Cocker Spaniel (each n=3), Poodle, The Schnauzer (each n=2), Border collie, Fox-terrier, Aberdeen terrier, Jack Russel terrier, and Thailand ridgeback (each n=1).

### CEA and CA 15-3 values

Based on the measured values in our preliminary study, we expect the following norms: for antigen CEA: 0.0–0.23 ng/ml, and for antigen CA 15-3: 0.0–7.00 IU/ml. However, in this study, a kit for CA 15-3 determination from another company was used because the previous kit was no longer produced. The new norm for CA 15-3 is 0.00-1.60 IU/ml.

The mean values of determined markers CEA and CA 15-3 are given in Table 3.

**Table 3.** Results of CEA (ng/ml) and CA 15-3 (IU/ml) tumor markers in dogs with mammary gland tumors (n=50) compared to control dogs (n=150)

| | CEA | CEA | CA 15-3 | CA 15-3 |
|---|---|---|---|---|
| | Control group (n=150) | Dogs with mammary tumors (n=50) | Control group (n=150) | Dogs with mammary tumors (n=50) |
| Mean | 0.89 | 1.53 | 1.52 | 2.87 |
| Std. Deviation | 0.79 | 1.15 | 0.66 | 1.11 |
| Minimum | 0.10 | 0.11 | 0.50 | 1.27 |
| Median | 0.715 | 1.395 | 1.3 | 2.69 |
| Maximum | 4.57 | 3.50 | 5.40 | 5.87 |
| Significance | | P<0.0001 | | P<0.0001 |

The mean values of the carcinoembryonic antigen markers ± SD were as follows: control group 0.89 ± 0.79 (min 0.1 – max 4.57), group with mammary gland tumor 1.53 ± 1.15 (min 0.11 – max 3.5). We found a significant difference between the group with mammary gland tumor and the control group ($P < 0.0001$****).

The values of cancer antigen markers CA 15-3 ± SD were: 1.52 ± 0.66 (min 0.5 – max 5.4) and 2.87 ± 1.11 (min 1.27 – max 5.87), respectively. The cancer antigen CA 15-3 values between groups were also statistically significant with $P < 0.0001$****.

## DISCUSSION

The determination of tumor markers in human medicine is considered a routine procedure for diagnostics and in particular for monitoring oncological diseases. CEA and CA 15-3 are the most thoroughly investigated serum tumor markers in breast cancer. Their use for early detection of metastases in bitches seems to be promising and their use for measuring the therapeutic response in metastatic disease is widely accepted. The cut-off values depend on many factors – for example, the antibody used, concentration, determination methods, and other analytical characteristics of the method [8]. In general terms, tumor markers cannot be used for primary diagnosis of breast cancer in women, as they are not specific and sensitive enough [16]. They seem to be promising and highly acceptable for early detection of metastatic processes, especially when they respond to treatment. Many of the studies have attempted to

assess predictive values of these markers. However, these analyses were performed with small samples of patients, focused only on a short time interval, and were, in a sense, evaluated from a one-sided perspective [17]. The choice of analytical methods is also influenced by the requirements of the diagnostic aim. Generally, the dog represents an optimal model organism to study cancer biology in a comparative setting, as many genes represent a great degree of homology to their human counterparts. To date, not many studies have tested both tumor markers CEA and CA 15-3 for independent prognostic value at the time of primary intervention in breast cancer animal patients [6,9]. Based on our preliminary study, we determined CEA and CA 15-3 levels in healthy bitches and bitches with mammary gland tumors. The results show that the tumor markers are present in a higher concentration in the group with mammary gland tumors compared to the healthy group. It is in correlation with results from human clinical studies [18,19]. In a study by Ramadan et al. (2022) [20]. CA 15-3 was elevated in all animals, whereas CEA levels showed no change compared to controls. However, in our study, the values of the markers were higher, especially in case of a progressive diagnosis. Jain et al. (2021) [21] and Fan et al. (2021) [22] demonstrated that CA 15-3 was found to be more sensitive than CEA, but detection of both will increase sensitivity. This determines the possibility of using both markers in the assessment of tumors in bitches.

Stieber (1996) [23] reported in a study of breast carcinoma in women that marker values of the majority of their patients were higher as compared with healthy women. Preoperative values of tumor markers in the serum could indicate an unfavorable prognosis [24]. Our results correlate with histological findings, providing a good strategy for the detection of mammary cancer in dogs. CA 15-3 is the main marker for monitoring oncological disease and CEA is a subsidiary marker in breast cancer. The increase in CA 15-3 with increasing stage of the disease was statistically significant, but no statistically significant correlation was observed between the stage of the disease and CEA's [25]. According to Marchesi et al. (2010) [26], CEA was not measured in all their samples by using the chemiluminescence method. The determination of CA 15-3 is apparently unable to differentiate between groups of clinically healthy dogs, dogs with non-neoplastic diseases, and dogs with tumor lesions by using the same method. CEA and CA 15-3 levels in bitches with mammary gland tumor determined in the present study were significantly higher compared to healthy bitches ($P < 0.0001$). In Pinheiro et al. (2023) [27] study, female dogs with canine mammary neoplasms were found to exhibit highest serum levels of CA 15-3. The authors of this study attempted to extend currently known diagnostic procedures of canine mammary gland tumors by introducing a new non-invasive method that effectively can be used before the onset of clinical signs, or for the purpose of postoperative diagnostics, if total bilateral mastectomy is not performed. The authors were inspired to investigate this problem by owners of bitches suffering from mammary gland cancer. When compared with values observed in human patients, CEA and CA 15-3 values observed in the present study were lower than the standards set by the INMM by 10% and 20%, respectively.

It can be argued that increased preoperative CEA and CA 15-3 values may indicate mammary gland cancer. Increased CA 15-3 values rather indicate alteration of tumor mass size due to previous changes in cell division activity [28] and serve as an important predictor of mammary gland tumors in bitches. The result of the study realized by Manuali et al. (2012) [8] reveal that CA 15-3 is expressed in both canine mammary tumor cell lines and tissues, and that serum levels significantly correlate with the histological grade of malignancy. However, marker levels can also increase without any association with mammary gland tumors. The specificity of both test methods was 95%.

## CONCLUSION

The analysis of oncomarkers is a valuable tool for practitioners in small animal oncology, having an advantage over tissue biomarkers as the measurement procedure is non-invasive and shows dynamic changes of physiological and pathological states before clinical signs appear. Determination of tumor markers in body fluids is one possibility for monitoring cancers. Detection levels of tumor markers CEA and CA 15-3 in bitches and they elaboration by the suitable method of analysis data leads to rapid determination of diagnosis, making it relevant in clinical practice and a promising prospect for the future.

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**Authors' contributions**

AV and LH drafted the manuscript and performed surgical procedures. BKB determinate tumor markers. MF made preoperative radiological examinations. SH made language corrections. All authors read and approved the final manuscript.

**Declaration of conflicting interests**

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

**Statement of Informed Consent**

The owner understood procedure and agrees that results related to investigation or treatment of their companion animals, could be published in Scientific Journal Acta Veterinaria-Beograd.

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## TUMOR MARKERI KOD PASA SA TUMORIMA MLEČNE ŽLEZDE

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Cilj istraživanja je bio da se utvrde i uporede vrednosti karcinoembrionalnog antigena (CEA) i antigena karcinoma (CA 15-3) kod 50 kuja sa tumorima dojke i 150 klinički zdravih pasa. Korišćena je modifikovana procedura za određivanje markera CEA i CA 15-3 sa humanim kit-ovima korišćenjem metode radioimunoeseja (RIA). Uzorci

prikupljeni iz ekstirpiranih tumora mlečnih žlezda su histološki obrađeni i klasifikovani prema smernicama SZO. Srednje vrednosti markera karcinoembrionalnog antigena ± SD bile su sledeće: kontrolna grupa 0,89 ± 0,79, grupa sa tumorom mlečne žlezde 1,53 ± 1,15. Vrednosti markera antigena karcinoma CA 15-3 ± SD bile su: 1,52 ± 0,66 i 2,87 ± 1,11, respektivno. Statistička značajnost markera karcinoembrionalnog antigena između grupa bila je $P < 0,0001$. Vrednosti antigena kancera CA 15-3 između grupa su takođe bile statistički značajne ($P < 0,0001$). Rezultati ove studije pokazuju da postoje značajne razlike u oba antigena između kontrolne grupe i grupa sa tumorom mlečne žlezde kod pasa.