



## Original Full Paper

# Canine mixed tumors as one of the mammary neoplasia varieties

Vasilisa Vasilievna Mitenko<sup>1\*</sup>, Olga Vladimirovna Dilekova<sup>1</sup>, Dmitriy Benikovich Galustyan<sup>1</sup>,  
Valentina Mikhaylovna Shpygova<sup>1</sup>, Victor Vasilievich Mikhailenko<sup>1</sup>

Federal State Budgetary Educational Institution of Higher Education Stavropol State Agrarian University, lane Zootechnichesky, 12, Stavropol, Russia

\*Corresponding author: [mitenko.vas@yandex.ru](mailto:mitenko.vas@yandex.ru)

Submitted: February 7<sup>th</sup>, 2023. Accepted: May 18<sup>th</sup>, 2023.

### Abstract

The most significant interest in veterinary oncology is occupied by tumors of a mixed nature, which are represented by the direct interaction of epithelial and mesenchymal components. According to some data, the formation of cell lineage of mesenchymal cells occurs due to the transformation of epidermal basket cells surrounding the glandular epithelium. In this case, the formation of a cartilage anlage with its further transformation into differentiated bone tissue occurs. The article contains information about the structure and features of the location and interaction of mixed tumors' glandular and stromal components in female dogs (n=29). It was revealed that in addition to simple mixed neoplasms, tumors with proplasia of the mesenchymal component into highly differentiated cartilage or bone tissue were also often registered. To confirm subcellular changes in the expression of certain types of proteins, such as  $\alpha$ -SMA and vimentin, an IHC study was used. The expression of the studied biomarkers was established in mesenchymal fibroblastic differon cells and myoepithelial cells. The intensity of immunoreactive material expression ranged from moderate (2+) to strong (3+), indicating changes in myoepithelial cells' genotype during the formation of mixed mammary neoplasms.

**Keywords:** dog, tumor, myoepithelial cells, immunohistochemistry (IHC), vimentin,  $\alpha$ -SMA.

### Introduction

The World Health Organization (WHO) published the "International Histological Classification of Pet Tumors" (18), in which mixed tumors are considered a complex histological picture, including epithelial and mesenchymal components. These tumors consist of innocent ductal and acinar epithelial structures under which myoepithelial cells are located. Another cell type is represented by mesenchymal cell lineage, which forms isogenous groups of chondrocytes in myxoid tissue, followed by the formation of a bone structure (2, 8, 11, 16, 19).

According to some data, myoepithelial cells begin to proliferate in the area between the epithelium and the basal membrane, they change their form and become more plastic and mobile, which allows them to penetrate the extracellular matrix through the basement membrane. Due to this activity, a nodular spindle cell mass arises, in which a type II collagen-containing cartilage matrix begins to be secreted and accumulated (1). For this rearrangement, in myoepithelial cells,

the change in cytoskeletal proteins occurs, the expression of basal cytokeratins (CK5, CK14, and CK17) decreases, and the expression of mesenchymal proteins such as  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and vimentin increases (4, 9, 13, 14, 15).

Modifying proteins at the molecular level leads to epithelial-mesenchymal transition (EMT), which stimulates myoepithelial cells to invade through the basement membrane and mediates changes in these cells into a mobile mesenchymal type. Such changes contribute to transforming the epithelial and stromal components within breast tumors (3).

The work aimed to study the morphological and molecular features of the stromal component of mixed mammary tumors in dogs.

### Materials and Methods

Female dogs were evaluated before the surgery (mastectomy) at the Scientific Diagnostic and Veterinary Treatment Center of Stavropol (Russia). Then the biopsy

specimens obtained during the operation were transferred to the Department of Parasitology and Veterinary Sanitary Expertise, Anatomy, and Pathoanatomy, named after Professor S.N. Nikolsky FSBEI HE “Stavropol State Agrarian University”. A confidentiality and informed consent form that allowed to research the submitted material was required for submission to the institutions mentioned above. Samples were not collected intentionally for this study and were submitted by veterinary clinicians between September 2019 and December 2022 after surgery (n=29) as a therapeutic intervention. Thus, the study did not require additional ethical approval.

For the histopathological study, 1cm<sup>3</sup> pieces of altered areas of the mammary glands were selected with the capture of healthy tissue and skin. The pieces were fixed for 48 hours in 10% buffered formalin (BioVitrum, Russia). The processing and filling of the material was carried out according to the generally accepted histological technique. From the obtained blocks, paraffin sections of mammary tumor tissue samples with a thickness of 3-4 microns were made, and mounted on glass. Next, standard staining with hematoxylin and eosin (H&E) and Mallory (BioVitrum, Russia) was performed on an automatic Prisma™ multistainer (Bio-Optica, Italy).

To determine vimentin localization, monoclonal rabbit antibodies Vimentin (SP20) 1:25 – 1:50 (USA, “Richard-Allan ScientificCo”) were used, localization of alpha-smooth muscle actin - monoclonal mouse antibodies (Actin, Smooth Muscle Ab-1 (1A4) 1:25 – 1:50 (USA, “Richard-Allan ScientificCo”). The slices were incubated for 20 minutes in a 3% hydrogen peroxide solution to block endogenous peroxidase. The formulation of IHC reactions was carried out using a peroxidase-polymer imaging system according to the manufacturer’s standard protocol (Dako, USA). The antibodies were unmasked by boiling the slices at 100 °C in a citrate buffer (pH = 6.0) for 10 min. At the final stage of the reaction, the slices were finished with Mayer’s hematoxylin. Reactions with replacing the first antibodies with dilution solution (SpringBioScience, USA) served as a negative control.

Microscopy of histological preparations was performed on an Olympus BX53 microscope with a built-in SC 50 camera (Japan). Ten digital images were taken from each preparation in immunopositive areas with magnification ×40, ×100, ×200, ×400, ×600, ×1000. The classification by M. Goldschmidt (2011) was used for the morphological assessment of mammary gland tumors in dogs.

The intensity of immunoreactive material expression was assessed visually, considering the percentage of active cells and the sum of immunopositive structures area according to the recommendations of the American Society of Clinical Oncology / College of American Pathologists (ASCO/CAP 2013):

1. Positive immunoreactivity (IHC 3+) if peripheral membrane full intense staining was observed in more than 10% of tumor cells.
2. Indeterminate immunoreactivity (IHC 2+) if there is a weak to moderate intensity, complete membrane staining of more than 10% of tumor cells.

3. Negative immunoreactivity (IHC 1+) if there is incomplete, weak/barely noticeable membrane staining of more than 10% of tumor cells.
4. Negative immunoreactivity (IHC 0) if no staining is observed or incomplete, weak/barely noticeable membrane staining of 10% or less of the tumor cells.

## Results

The age, breed, invaded mammary gland, and tumor tissue components of the studied dogs are presented in Table 1. According to the results of the pathohistological examination of mammary tumors, samples from dogs were diagnosed as mixed tumor (n=10) – 34%, mixed tumor with cartilaginous metaplasia (n=9) – 31%, mixed tumor with bone metaplasia (n=5) – 17.2%, mixed tumor with transformation into adenocarcinoma (n=4) – 13.8%, mixed tumor with transformation into carcinoma (n=1) – 3.4%.

Simple mixed tumors have the highest percentage. When assessing histological sections, tissue structures represented by epithelial and mesenchymal components were revealed. The epithelial component was located in the tumor tissue in the form of islands surrounded by fusiform basket cells. According to the phenotype, epithelial cells were monomorphic, prismatic, and arranged in 1-2 layers. The epithelium of the islets creates the appearance of papillary structures anastomosing with each other. The mesenchymal component has a nodular structure. The nodes include loosely located fibroblastic differon cells, between which a scanty multidirectional connective tissue was placed (Fig. 1).

Mixed tumors with metaplasia into cartilage tissue occupy an intermediate position among other neoplasm types. The morphological picture was represented by developing a moderately pleomorphic epithelial component, from a cubic to a low-prismatic form of cells arranged in 2-3 layers. Myoepithelial cells of elongated, fusiform shape were located under them. The mesenchymal component was strongly developed concerning the epithelial one, and there was a prosoplastic metaplasia of fibroblastic differon cells into young chondrocytes. The cells were arranged singly or with the formation of isogenic groups in the amount of 2 to 4 chondrocytes localized in an abundant extracellular matrix, having a color from light basophilic to dark, which was characterized by the presence of acidic proteoglycans in it (Fig. 2).

The next type of dog neoplasm was a mixed tumor with bone metaplasia. The epithelial component forms pleomorphic, tubular, and papillary structures, the cells of which have a shape from cubic to low-prismatic, arranged in 2-3 layers. Basket cells were located under the epithelial lining, appearing as elongated spindle-shaped structures. Individual cells next to the cartilaginous skeleton have a light cytoplasm with a swollen nucleus.

Metaplasia occurs due to elongated fibroblastic differon cells with numerous processes that form a nodular

**Table 1.** Age, breed, invaded mammary gland, and tumor tissue components of the studied dogs.

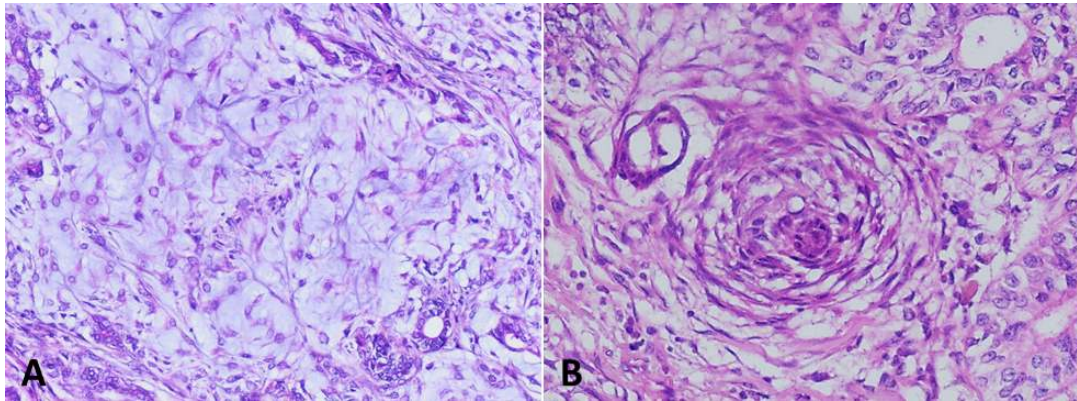
Dog No.	Age	Breed	Invaded mammary gland	Tumor tissue components
1	9 years	crossbred	4 pack	bone tissue
2	14 years	Poodle	4 pack	bone tissue
3	13 years	Labrador	3 pack	bone tissue
4	7 years	Russian Spaniel	4 pack	bone tissue
5	7 years	Yorkshire Terrier	4 pack	bone tissue
6	9 years	crossbred	5 pack	cartilaginous tissue
7	12 years	Dachshund	4 pack	cartilaginous tissue
8	9 years	Zwergpinscher	5 pack	cartilaginous tissue
9	10 years	Dalmatian	5 pack	cartilaginous tissue
10	13 years	Deutsch Boxer	3 pack	cartilaginous tissue
11	9 years	Yorkshire Terrier	5 pack	cartilaginous tissue
12	8 years	Jack Russell Terrier	2 pack	cartilaginous tissue
13	7 years	Yorkshire terrier	5 pack	cartilaginous tissue
14	7 years	crossbred	4 pack	cartilaginous tissue
15	9 years	crossbred	3 pack	myxoid tissue
16	7 years	Pommeraner Spitz	5 pack	myxoid tissue
17	8 years	Yorkshire Terrier	4 pack	myxoid tissue
18	13 years	Yorkshire Terrier	3 pack	myxoid tissue
19	9 years	crossbred	3 pack	myxoid tissue
20	10 years	crossbred	3 pack	myxoid tissue
21	9 years	Pommeraner Spitz	4 pack	myxoid tissue
22	8 years	Shar-Pei	4 pack	myxoid tissue
23	10 years	Dachshund	3 pack	myxoid tissue
24	5 years	Yorkshire Terrier	2 pack	myxoid tissue
25	10 years	Yorkshire Terrier	3 pack	epithelial tissue → adenocarcinoma
26	9 years	crossbred	4 pack	epithelial tissue → adenocarcinoma
27	12 years	Yorkshire Terrier	3 pack	epithelial tissue → adenocarcinoma
28	10 years	crossbred	4 pack	epithelial tissue → adenocarcinoma
29	14 years	crossbred	5 pack	epithelial tissue → carcinoma

structure of myxoid connective tissue in the center. In some areas, prosoplastic metaplasia of fibroblastic cells into young chondrocytes takes place, forming a basophilic extracellular matrix. The appearance of osteoclasts in the state of phagocytosis of cartilage structures was registered, which leads to its resorption. In addition to osteoclasts, numerous osteoblasts were visualized on the periphery of these structures, which leads to bone restructuring and structuring of individual cartilage sections into bone trabeculae.

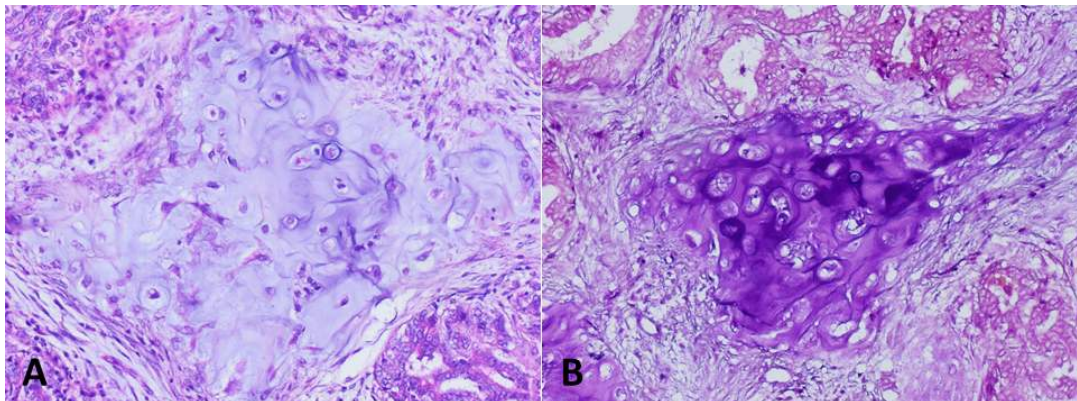
The young bone matrix has a light basophilic color, and highly differentiated bone tissue was characterized by pronounced metachromasia. In the mature bone trabeculae,

reticulofibrous tissue was formed, in which the laying of myeloid cells was visualized. We believe these structural, morphological transformations reflect the inherent genetic determination of mesenchymal cells in mammary tumor tissues (Fig. 3).

Immunohistological examination (IHC) showed that the expression of vimentin in all samples was observed from moderate (2+) to strong (3+) and was located in fibroblastic different and myoepithelial cells (Table 2). Immunopositive myoepithelial cells had an elongated fusiform shape under the epithelial layer in discontinuous chains. The marker expression pattern was localized in the cytoplasm in the form



**Figure 1.** Mixed tumor. A – mesenchymal tumor component. Dog, crossbred, eight years (stained with hematoxylin and eosin, mag.  $\times 100$ ). B - Fibroblastic structures, looking like a vortex. Dog, St. Bernard, eight years (stained with hematoxylin and eosin, mag.  $\times 200$ ).



**Figure 2.** Mixed tumor with cartilaginous metaplasia. A – mesenchymal component – hyaline cartilage. Dog, crossbred, eight years (stained with hematoxylin and eosin, mag.  $\times 200$ ). B – hyaline cartilage with isogenous groups of chondrocytes (arrow). Dog, crossbred, eight years (Mallory staining, mag.  $\times 100$ ).

of sprayed light brown granules or a perinuclear rim of dark brown granules.

The expression of  $\alpha$ -SMA in myoepithelial cells was observed from moderate (2+) to strong (3+) in all samples. The biomarker localization in immunopositive cells was light brown grains of a crumbly structure throughout the cytoplasm. It was noted that the cells with pronounced overexpression were located next to the cartilage/bone structure (Fig. 4).

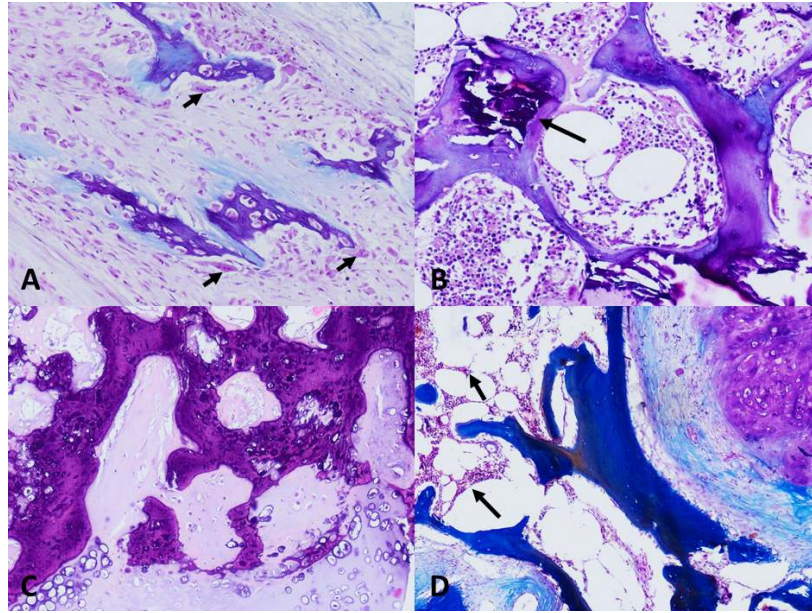
## Discussion

Canine mammary tumors (CMT) are the most common oncological disease pathology; more than 50% are malignant neoplasms. In animals, neoplasias are most often recorded at the age of 7, in most cases in uncastrated females (12).

Breed susceptibility in dogs was not thoroughly studied, but according to some data, small and large dog breeds are most susceptible to the development of mammary gland tumors: Chihuahua, Dachshund, Poodle, Yorkshire Terrier, Bulldog, Spaniel, German Shepherd, Doberman, German Boxer (7).

Pathologists use the classification of M. Goldschmidt et al. (8) when assessing the morphological type of tumors in dogs in a new format through the revision of the WHO CMTs classifications of 1974 and 1999. In addition to the main terms of mammary gland tumors, another one was added - inflammatory carcinoma, having clinical signs with inflammatory reactions of the body (8).

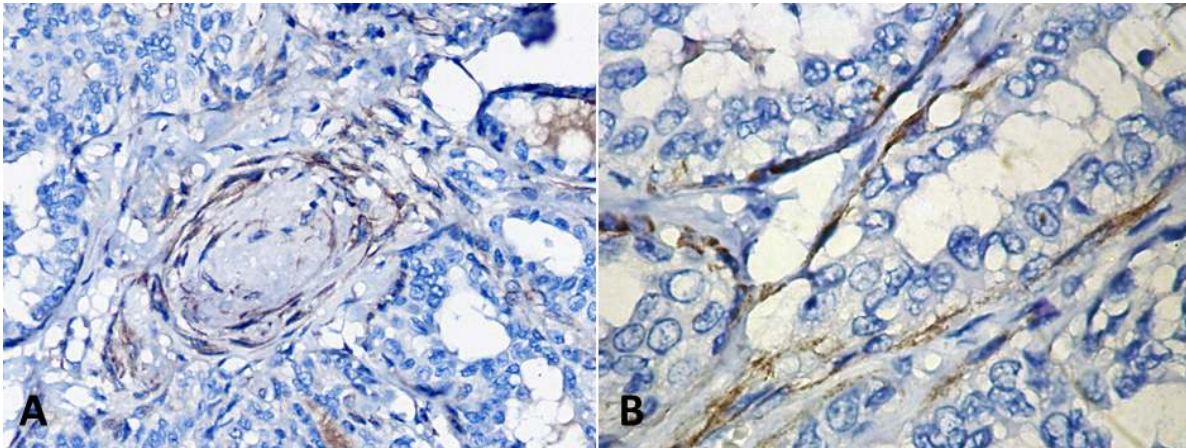
In addition to the morphological classification in 2014, it was decided to supplement it with the use of immunohistochemical study (IHC) for markers p63,  $\alpha$ -SMA, and



**Figure 3.** Mixed tumors with bone metaplasia. A – Bone tissue anlage and osteoclasts are nearby (arrows). Dog, Poodle, 14 years (Mallory staining, mag.  $\times 100$ ). B – Bone tissue trabecula mineralization (arrows). Dog, crossbred, ten years (stained with hematoxylin and eosin, mag.  $\times 100$ ). C – Transformation of hyaline cartilage into bone tissue. Dog, Chinese Crested, nine years (stained with hematoxylin and eosin, mag.  $\times 100$ ). D – Trabecular bone tissue with the formation of myeloid-type cells between the trabeculae (arrows). Dog, Russian Spaniel, 7,5 years (stained with hematoxylin and eosin, mag.  $\times 40$ ).

**Table 2.** Indicators of immunoreactivity in the myoepithelial component of tumors.

Dog No.	Age	Breed	Tumor tissue components	Immunoreactivity
1	9 years	crossbred	bone tissue	$\alpha$ -SMA (+++) vimentin (+++)
2	14 years	Poodle	bone tissue	$\alpha$ -SMA (+++) vimentin (+++)
3	13 years	Labrador	bone tissue	$\alpha$ -SMA (+++) vimentin (+++)
4	7 years	Russian Spaniel	bone tissue	$\alpha$ -SMA (+++) vimentin (+++)
5	7 years	Yorkshire Terrier	bone tissue	$\alpha$ -SMA (+++) vimentin (+++)
6	9 years	crossbred	cartilaginous tissue	$\alpha$ -SMA (+++) vimentin (++)
7	12 years	Dachshund	cartilaginous tissue	$\alpha$ -SMA (+++) vimentin (+++)
8	9 years	Zwergpinscher	cartilaginous tissue	$\alpha$ -SMA (+++) vimentin (+++)
9	10 years	Dalmatian	cartilaginous tissue	$\alpha$ -SMA (+++) vimentin (+++)
10	13 years	Deutsch Boxer	cartilaginous tissue	$\alpha$ -SMA (+++) vimentin (+++)
11	9 years	Yorkshire Terrier	cartilaginous tissue	$\alpha$ -SMA (++) vimentin (+++)
12	8 years	Jack Russell Terrier	cartilaginous tissue	$\alpha$ -SMA (+++) vimentin (+++)
13	7 years	Yorkshire Terrier	cartilaginous tissue	$\alpha$ -SMA (+++) vimentin (+++)
14	7 years	crossbred	cartilaginous tissue	$\alpha$ -SMA (+++) vimentin (+++)
15	9 years	crossbred	myxoid tissue	$\alpha$ -SMA (++) vimentin (++)
16	7 years	Pommeranter Spitz	myxoid tissue	$\alpha$ -SMA (+++) vimentin (++)
17	8 years	Yorkshire Terrier	myxoid tissue	$\alpha$ -SMA (++) vimentin (+++)
18	13 years	Yorkshire Terrier	myxoid tissue	$\alpha$ -SMA (++) vimentin (++)
19	9 years	crossbred	myxoid tissue	$\alpha$ -SMA (++) vimentin (+++)
20	10 years	crossbred	myxoid tissue	$\alpha$ -SMA (++) vimentin (++)
21	9 years	Pommeranter Spitz	myxoid tissue	$\alpha$ -SMA (+++) vimentin (++)
22	8 years	Shar-Pei	myxoid tissue	$\alpha$ -SMA (++) vimentin (++)
23	10 years	Dachshund	myxoid tissue	$\alpha$ -SMA (+++) vimentin (++)
24	5 years	Yorkshire Terrier	myxoid tissue	$\alpha$ -SMA (++) vimentin (+++)



**Figure 4.** Biomarker expression. A – vimentin<sup>+</sup> expression in myoepithelial cells. Mixed tumor with cartilaginous metaplasia, dog, crossbred, eight years (mag. ×400). B –  $\alpha$ -SMA<sup>+</sup> expression in myoepithelial cells. Mixed tumor with bone metaplasia, dog, Poodle, 13 years (mag. ×1000). Immunohistochemical reaction with antibodies to vimentin/ $\alpha$ -SMA. Mayer's hematoxylin nucleus staining.

vimentin, for further identification of cellular structures derived from myoepithelial cell lineage (10).

In our study, mixed tumors with a complicated cellular landscape of epithelial, myoepithelial, and mesenchymal components represented a common type of canine mammary neoplasia. There was no consensus on the origin of such diverse cellular structures in tumors, and only three hypotheses were presented. The first one refers to the collision theory when two tumors begin to unite their cellular components due to their proximity. The second one was the combination theory, which indicates that the origin of the three tissues begins with their stem or multipotent cells. The last one refers to the conversion (metaplastic) theory, based on metaplasia and transdifferentiation of myoepithelial or basal mammary gland cells (16, 17).

For further study of cellular elements in mixed mammary gland tumors, an immunohistochemical study of versican proteoglycan was used, which proves molecular changes in myoepithelial cells, leading to transformation into a mesenchymal component, which is the basis for the cartilaginous matrix formation (5, 6).

We can conclude that among mixed mammary tumors in dogs, the most significant percentage is occupied by neoplasms with cartilaginous and bone metaplasia. The pathognomic morphological feature is the nodular structuring of fibroblastic lineage cells, with further transformation into hyaline cartilage tissue, which undergoes remodeling to a highly differentiated bone structure. An immunohistochemical study showed that the biomarkers - vimentin,  $\alpha$ -SMA, have cytoplasmic immunoreactivity in myoepitheliocytes. In our opinion, the presence of hyperexpression indicates the intercellular effect of myoepithelial cells on the mesenchymal component, which contributes to the prosoplastic metaplasia activation inside the tumor tissue.

#### Acknowledgements

This study was sponsored within the framework of the grant of the All-Russian competition “UMNIK-2020” (contract No. 16027GU/2020 dated December 24th, 2020), topic “Development of a method for determining the FGFR1 gene status in mammary gland cancer using fluorescent in situ hybridization”.

#### Conflict of Interest

The authors declare no competing interests.

#### References

1. Arai K, Nakano H, Matsuda H. Expression of class II  $\beta$ -tubulin by proliferative myoepithelial cells in canine mammary mixed tumors. *Vet Pathol.* 2003;40(6):670-6.
2. Cassali G, Bertagnolli A, Ferreira E, Damasceno K, Conrado de Oliveira G, Bonolo de Campos C. Canine mammary mixed tumours: a review. *Vet Med Int.* 2012;2012:274608.
3. Chen T, You Y, Jiang H, Wang Z. Epithelial-mesenchymal transition (EMT): a biological process in the development, stem cell differentiation and tumorigenesis. *J Cell Physiol.* 2017;232(12):3261-72.
4. Chenab Z, Fangab Z, Maa J. Regulatory mechanisms and clinical significance of vimentin in breast cancer. *Biomed Pharmacother.* 2021;133:111068.
5. Damasceno KA, Bertagnolli AC, Estrela-Lima A, Ribeiro LG, Rabelo BS, Campos CB, Barros AL, Cassali GD. Versican expression in canine carcinomas of benign

- mixed tumors: is there a relationship with clinical pathological factors, invasion and overall survival? *BMC Vet Res.* 2012;8:195-212.
6. Damasceno KA, Bertagnolli AS, Estrella-Lima A, Rabelo BS, Campos LC, Ribeiro LG, Cassali GD. Versican expression in myoepithelial cells from carcinomas in canine mixed mammary tumors. *Vet J.* 2014;200(1):146-51.
  7. Dobson JM, Lascelles BDX. *BSAVA Manual of Canine and Feline Oncology.* 3rd ed. Gloucester: BSAVA, 2011. 364p.
  8. Goldschmidt M, Peña L, Zappulli V. Classification and grading of canine mammary tumors. *Vet Pathol.* 2011;48(1):117-31.
  9. Hinz B, Celetta G, Tomasek J, Gabbiani G, Chaponnier C. Alpha-smooth muscle actin expression upregulates fibroblast contractile activity. *Mol Biol Cell.* 2001;12(9):2730-41.
  10. Im KS, Kim NH, Lim HY, Kim HW, Shin JI, Sur JH. Analysis of a new histological and molecular-based classification of canine mammary neoplasia. *Vet Pathol.* 2014;51(3):549-59.
  11. Kiyoshi T, Uchida K, Tateyama S. Expression of bone morphogenetic protein-6 and bone morphogenetic protein receptors in myoepithelial cells of canine mammary gland tumors. *Vet Pathol.* 2004;41(2):154-63.
  12. Misdorp W, Meuten DJ. Mammary Tumors. In: Meuten DJ, editor. *Tumors in Domestic Animals.* 4th ed. Ames: Iowa State Press. 2002. p.575-88.
  13. Muchlińska A, Nagel A, Popęda M, Szade M, Niemira M, Zieliński J, Skokowski J, Bednarz-Knoll N, J Żaczek A. Alpha-smooth muscle actin-positive cancer-associated fibroblasts secreting osteopontin promote growth of luminal breast cancer. *Cell Mol Biol Lett.* 2022;27(1):45.
  14. Paulin D, Lilienbaum A, Kardjian S, Agbulut O, Li Z. Vimentin: regulation and pathogenesis *Biochimie.* 2022;197:96-112.
  15. Sachiyo N. Identification, friend or foe: vimentin and  $\alpha$ -smooth muscle actin in cancer-associated fibroblasts. *Ann Surg Oncol.* 2019;26(13):4191-2.
  16. Sánchez Céspedes R, Millána Y, Guil-Lunaa S, Reymundob C, Espinosa de los Monteros A, Martín de las Mulasa J. Myoepithelial cells in canine mammary tumours. *Vet J.* 2016;207:45-52.
  17. Sánchez-Céspedes R, Maniscalco L, Iussich S, Martignani E, Guil-Luna S, De Maria R, Martín de Las Mulas J, Millán Y. Isolation, purification, culture and characterisation of myoepithelial cells from normal and neoplastic canine mammary glands using a magnetic-activated cell sorting separation system. *Vet J.* 2013 Aug;197(2):474-82.
  18. WHO - World Health Organization. *International Histological Classification of Tumors of Domestic Animals: Histological classification of mammary tumors of the dog and the cat.* Washington: Armed Forces Institute of Pathology, 1999. 59p.
  19. Yi-Hsuan H, Horng-Der T, Ming-Chih C and Yan-gao M. The myoepithelial cell layer may serve as a potential trigger factor for different outcomes of stage-matched invasive lobular and ductal breast cancers. *Int J Biol Sci.* 2011;7(2):147-53.