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RESEARCH ARTICLE

Immunohistochemical Evaluation of BRCA1 and BRCA2 Expressions in Dogs with Different Grades of Malignancy, Histological Subtypes and Metastatic/Non-Metastatic Mammary Gland Adenocarcinomas

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ABSTRACT

This study primarily aimed to examine BRCA1 and BRCA2 expression in various types of canine mammary gland adenocarcinomas using immunohistochemistry with the avidin-biotin-peroxidase Complex (ABC). The study material comprised adenocarcinoma samples obtained from the mammary glands of 20 dogs that were presented for routine histopathological examination at the Department of Pathology in the Faculty of Veterinary Medicine at Kafkas University between 2012 and 2022. The tumour masses had a highly haemorrhagic and ulcerative appearance. These round or oval-shaped masses were primarily greyish-white in colour. Especially in mixed-type carcinomas, tumour masses were particularly firm in consistency due to the presence of bone and cartilage components. 5 out of 20 cases (25%) were classified as mixed-type carcinoma, 4 out of 20 cases (20%) as tubular-type carcinoma, 4 out of 20 cases (20%) as solid-type carcinoma, 3 out of 20 cases (15%) as tubulopapillary-type carcinoma, 3 out of 20 cases (15%) as intraductal papillary-type carcinoma and 1 out of 20 cases (5%) as micropapillary-type carcinoma. 6 cases were classified as grade-1 malignancy (30%), 6 as grade 2 (30%), and the remaining 8 as grade 3 (40%). Metastatic tumour foci in the lung and lymph nodes were also confirmed by histopathology. No difference was observed between the degree of differentiation of secondary and primary tumour foci. BRCA1- and BRCA2-positive immunolabelling were predominantly concentrated in the cytoplasm of tumour cells. Compared to metastatic cases, the immunopositivity for the aforementioned biomarkers was significantly more intense in non-metastatic cases. However, BRCA1 and BRCA2 immunoreactivities were significantly higher in early-grade cases than in advanced-grade cases. Although an ideal biomarker for canine mammary gland tumours has yet to be identified, immunohistochemical data obtained from the current study on BRCA1 and BRCA2 are considered important and useful biomarkers for determining malignancy grade, prognosis, and metastatic capacity in canine mammary gland adenocarcinomas.

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INTRODUCTION

Although the cause of canine mammary tumours remains unclear, several significant risk factors have been identified, including environmental, hereditary, hormonal, nutritional, and genetic factors (Im *et al.*, 2013; Kaszak *et al.*, 2018). Among these risk factors, sex hormones are the leading contributors to tumour development (Kaszak *et al.*, 2022). Surgical resection is the most effective treatment method; however, complementary therapy is often ineffective (Cardama *et al.*, 2023). Similarities in the biology, epidemiology and genetics of canine and human mammary tumours suggest that dogs could be a valuable model for studying human breast cancer (Di Giacomo *et al.*, 2022; Petrouškova *et al.*, 2025). Owners most frequently notice mammary tumours in patients when the visible macroscopic changes in the mammary gland are caused (Thumser-Henner *et al.*, 2020). Since canine mammary tumours display a heterogeneous morphology and biological characteristics, selecting an effective and

reliable biomarker for the diagnosis is challenging (Jain *et al.*, 2021). Tumour size, stage, malignancy grade, histological type, lymph node involvement, distant tissue metastasis, and proliferation index are the parameters used to determine the prognosis of canine mammary tumours (Munday *et al.*, 2019; Sakalauskaitė *et al.*, 2021). Additionally, artificial intelligence (AI) has been used for diagnostic purposes (Choudhary, 2025; Choudhary *et al.,* 2025).

Canine mammary carcinoma has a distinct genetic susceptibility (Thumser-Henner *et al.*, 2020). BRCA1 and BRCA2 (Breast Cancer 1 and 2) are currently regarded as the most significant susceptibility genes associated with canine mammary carcinoma (Enginler *et al.*, 2014). The encoded proteins primarily repair double-strand DNA damage and have a crucial role in the regulation of cell-cycle and genomic stabilisation (Yang *et al.*, 2023). For this reason, they are regarded as the genomic caretakers (Klopfleisch and Gruber, 2009).

This study aimed to evaluate the expression of BRCA1 and BRCA2 in different types of canine mammary gland adenocarcinomas using immunohistochemical methods. The study evaluated the correlation between immunoreactivity of tumour biomarkers and varying malignancy grades and metastatic status. Furthermore, the effectiveness of these biomarkers in the prediction of prognosis, tumour aggressiveness, and disease progression was analysed using the obtained data.

MATERIALS AND METHODS

Animals: The study material consisted of biopsy samples obtained from the mammary glands of 20 dogs brought to the Department of Pathology at the Faculty of Veterinary Medicine at Kafkas University by the Department of Obstetrics and Gynaecology, as well as to private veterinary clinics for routine histopathological diagnosis following surgical procedures between 2012 and 2022. The average age of the dogs was 9.15 years. Among the female dogs, 9 were crossbreds, 8 were Kangal Shepherds, 2 were Setters, and 1 was a German Shepherd.

Ethics committee report: The study was approved by the Animal Experiments Local Ethics Committee of Kafkas University (Approval No: KAU-HADYEK/2024-111, Date: 05.06.2024).

Histopathological examinations: Following a systematic necropsy, biopsy samples were collected from the mammary glands and various internal organs suspected of metastasis (the lung, liver, uterus, spleen and lymph nodes). The samples were then fixed in 10% formaldehyde. Paraffin blocks were prepared, and serial sections that were 5 micrometres thick were obtained. These sections were incubated at 60°C for 60 minutes prior to staining with H&E. The steps in the staining procedure are as follows: 1. Xylene – (10 minutes), 2. Xylene – (10 minutes), 3. 100% Alcohol – (5 minutes), 4. 96% Alcohol – (3 minutes), 5. 90% Alcohol – (3 minutes), 6. 80% Alcohol – (3 minutes), 7. 70% Alcohol – (3 minutes), 8. Wash in Distilled Water – (3 minutes), 9. Hematoxylin – (10 minutes), 10. Wash in Tap Water – (1 minute), 11. Acid Alcohol (1%) – (15-20 seconds), 12. Wash in Distilled Water – (1 minute), 13. Ammonia Water (1%) – (5 minutes), 14. Wash in Distilled Water – (3 minutes), 15. Eosin (Alcoholic Based) – (3 minutes), 16. 96% Alcohol – (3 Minutes), 17. 100% Alcohol – (5 Minutes), 18. Xylene – (10 Minutes), 19. Xylene – (10 Minutes), 20. Closing with Entellan. The slides were thoroughly evaluated under a light microscope by at least two different pathologists. Photographs of the findings were captured and recorded (Feldman and Wolfe, 2014; Karakurt *et al.*, 2021)

Tumours were classified according to the criteria determined by Goldschmidt *et al.* (2011) and Goldschmidt *et al.* (2017). The grade of malignancy of the tumours was assessed using the Nottingham (Elston-Ellis) system adapted for dogs (Peña *et al.*, 2012) (Table 1). Table 2 presents details of the animals involved in the study, including the types of canine mammary gland adenocarcinoma and their respective malignancy grades.

Table 1: Criteria used for histological malignancy grading of canine mammary gland tumours

		Scoring
A-Tubule Formation	Tubular structure present in most of the tumours >%75	1
	Tubular structure with solid areas present in 10-75%	2
	Tubular structure present in <10% (minimal or no tubular structure)	3
B-Nuclear Pleomorphism	Nuclei are uniform, regular and small	1
	Nuclei are moderately differentiated, and nucleoli is visible in some places	2
	High differentiation, contains one or more prominent nucleoli	3
C-Mitosis Count	0-9/10 LMA	1
	10-19/ 10 LMA	2
	≥20/ 10 LMA	3
	Grades of Malignancy	
Total Score (A+B+C)		Grades
3-5		1
6-7		2
8-9		3

LMA: Large Magnification Area

Table 2: Tumour classification, malignancy grades and metastasis data for all cases

Case	Age-Year	Breed	Gender	Classification	Grade	Metastasis
1	10	Mixed	Female	Tubular Type	1	-
2	4	Kangal	Female	Mixed Type	2	-
3	15	Kangal	Female	Tubulopapillary Type	3	+
4	4	Setter	Female	Intraductular Papillary Type	2	-
5	17	Setter	Female	Solid Type	3	-
6	6	Kangal	Female	Mixed Type	1	-
7	10	Mixed	Female	Intraductular Papillary Type	1	-
8	4	Kangal	Female	Solid Type	3	+
9	9	Mixed	Female	Mixed Type	2	-
10	10	Mixed	Female	Mixed Type	1	-
11	4	Kangal	Female	Micro Papillary Type	2	-
12	9	Mixed	Female	Solid Type	3	+
13	11	Kangal	Female	Mixed Type	3	+
14	9	Mixed	Female	Solid Type	3	+
15	10	Mixed	Female	Intraductular Papillary Type	1	-
16	7	Mixed	Female	Tubulopapillary Type	3	+
17	8	Kangal	Female	Tubular Type	3	+
18	10	Mixed	Female	Tubular Type	2	-
19	10	Kangal	Female	Tubulopapillary Type	2	-
20	16	German Shepherd	Female	Tubular Type	1	-

Immunohistochemical examinations: Serial sections, each 4 micrometres thick, were obtained from paraffin-embedded

tumour tissues and subsequently labelled using the Avidin–Biotin Complex (ABC) peroxidase method, employing commercial antibodies for BRCA1 and BRCA2 in accordance with the manufacturer's guidelines. Details about the primary antibodies are listed in Table 2. Thermo Scientific Histostain Immunohistochemistry (IHC) Kit (HRP, broadspectrum, REF: TP-125-HL) was used for all immunolabelling. As a chromogenic substrate, 3.3-diaminobenzidine tetrahydrochloride (DAB) solution (Thermo Scientific, REF: TA-125-HD) was applied and allowed to incubate for 15 minutes. The sections were washed with distilled water for 5 minutes, stained with Mayer's Haematoxylin, and mounted using Immuno Mount.

Table 3: Information on primary antibodies used in immunohistochemical evaluations

Primary Antibodies	Company and Catalogue Numbers	Pre-treatment	Dilution	Incubation Conditions
BRCA 1	ABclonal, polyclonal A0212,	Microwave oven	1/100	Overnight, 4°C
BRCA 2	ABclonal, polyclonal A2435,	Microwave oven	1/100	Overnight, 4°C

Following the mounting procedure, the preparations were examined under a light microscope (Olympus Bx53) and photographs of the sections were captured using the Cell^P software (Olympus Soft Imaging Solutions GmbH, 3.4). The photographs were then analysed in detail using Image J software (1.51j8).

The results of the immunohistochemical labelling were evaluated using a grading system based on the number of positively stained cells (tumour cells, tumour microenvironment, etc.) in areas exhibiting the strongest immunopositive reactions. The quantification of immunopositive reactions in tissues was initiated by analysing regions exhibiting the highest staining intensity. Three separate fields within each tumour tissue were examined using a 40x lens. The number of positively labelled cells in each field was individually recorded, and the mean value across three fields was considered the representative positive cell count for each case (Beytut *et al.*, 2024).

Statistical analysis: Statistical analyses were performed utilising GraphPad Prism® software (Version 9.5.1, GraphPad Software Inc., San Diego, CA, USA). Intergroup differences were deemed statistically significant at $P<0.05$. A parametric two-group t-test was conducted to evaluate BRCA1 and BRCA2 expression levels between the metastasis and non-metastasis groups. A parametric one-way ANOVA followed by the Tukey's test was used to assess BRCA1 and BRCA2 expression values across Grade 1, 2, and 3 groups.

RESULTS

Macroscopic findings: Macroscopic examination revealed that the tumour masses exhibited a markedly haemorrhagic and ulcerative appearance. The masses were mainly round or oval. Although their colours varied, the tumour masses predominantly appeared greyish-white. In some cases, the masses were firm due to the presence of bone and cartilage. In some instances, the cross-sectional surfaces of the tumour formations were notably soft and spongy (Fig. 1A). Metastases were identified in only 7 cases (7 out of 20) in organs such as the lungs and lymph nodes. Of the aforementioned organs, the lung was the most prone to metastasis (Fig. 1C-D).

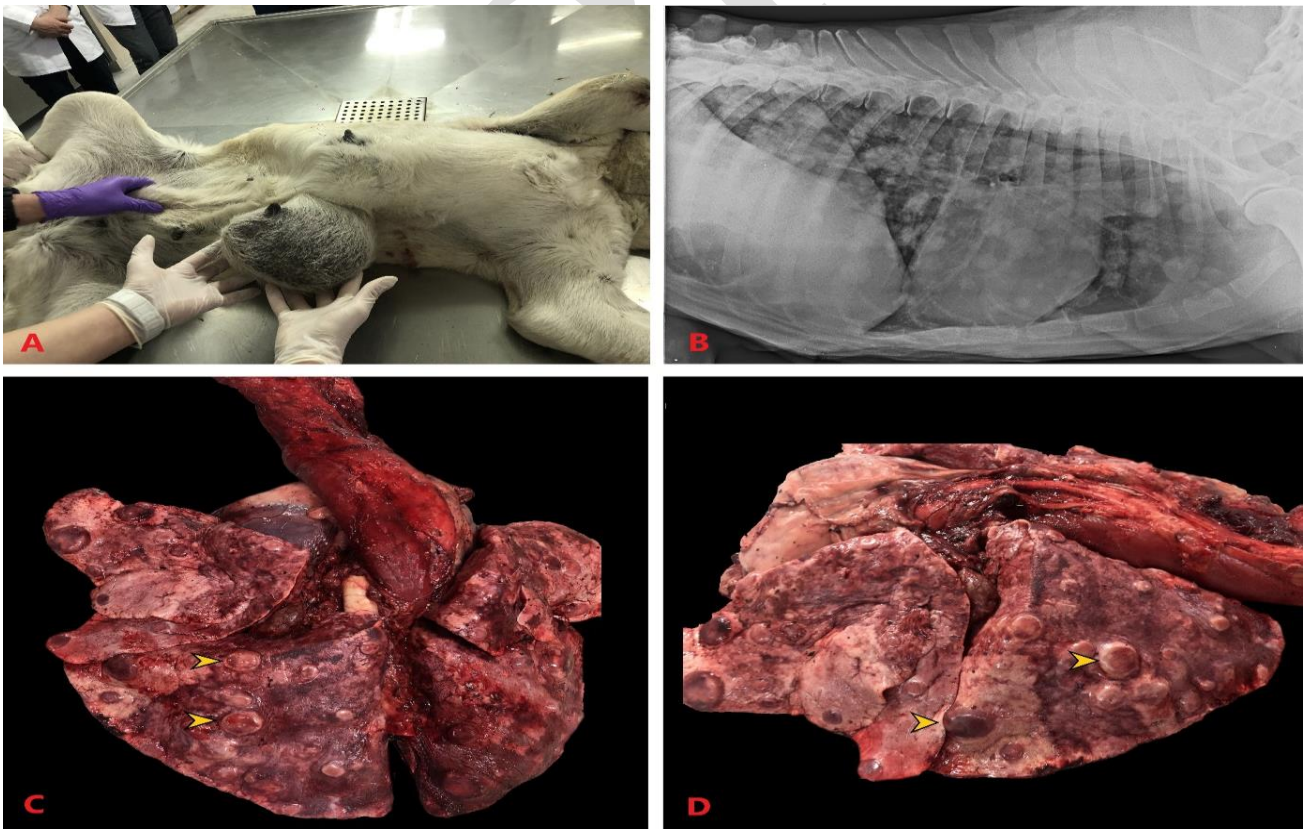

Fig. 1: Dog, **A:** Macroscopic view of the primary tumour mass, **B:** Thoracic radiograph, metastatic foci, **C-D:** Views from different angles of metastatic foci of varying sizes (yellow arrowheads) scattered across the lung surface.

Radiographic findings: Latero-lateral radiographic imaging revealed metastatic masses throughout the lungs, extending from the cranial to the caudal regions and from the dorsal to the ventral regions. Marked consolidation was present in the caudal region of the lung, gradually reducing towards the cranio-ventral part (Fig. 1B).

Microscopic findings: 5 out of 20 cases were classified as mixed-type carcinoma (25%), 4 out of 20 cases as tubular-type carcinoma (20%), 4 out of 20 cases as solid-type carcinoma (20%), 3 out of 20 cases as tubulopapillary-type carcinoma (15%), 3 out of 20 cases as intraductal papillary-type carcinoma (15%) and 1 out of 20 cases as micropapillary-type carcinoma (5%). In the mixed type, three different cell populations were observed: first, epithelial cells organised in irregular tubules; second, spindle-shaped myoepithelial cells; and third, foci of cartilage and/or bone. In tubular-type carcinomas, predominantly tubular or gland-like structures were seen, and the tubular lining was 1-2 cells thick. In the solid type, cells were organised predominantly into solid sheets without lumina, composed of irregularly sized lobules with supportive fibrovascular stroma. In tubulopapillary-type carcinomas, papillae extended into the tubular lumina and papillary structures. The papillae were supported by a fibrovascular stroma. In intraductal-type carcinomas, multilayer epithelial cells with malignant characteristics were observed. Additionally, papillae were supported by fibrous connective tissue and myoepithelial cells. For the micropapillary type, papillae were small and lacked a supporting fibrovascular stalk. Additionally, intraductal, intraluminal irregular aggregates were observed in mammary tissue nodules. 6 out of 20 cases were determined to be at malignancy Grade 1 (30%), 6 out of 20 cases were Grade 2 (30%) and the remaining 8 out of 20 cases were Grade 3 (40%) (Fig. 2). Metastatic tumour foci in the lung and lymph node were also confirmed by histopathology (n=7) (Fig. 3).

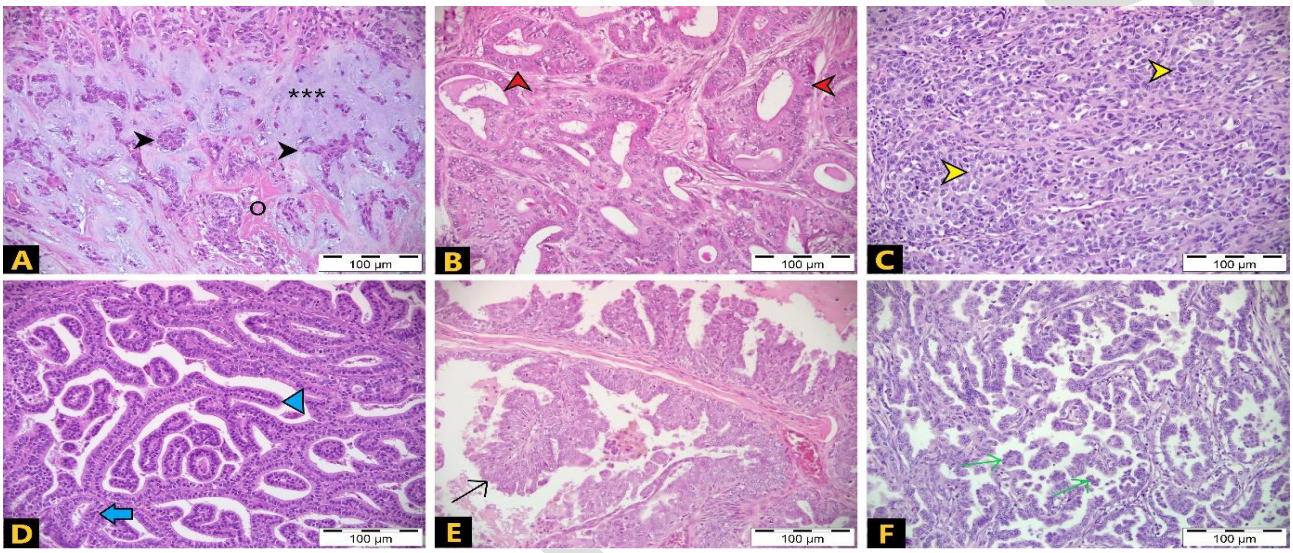

Fig. 2: Dog, mammary tissue, H&E, Bar= 100 microns **A:** Mixed-type carcinoma, malign epithelial component (black arrowheads), osteoid formations (O), cartilage formations (stars) **B:** Tubular-type carcinoma, 1-2 cell lines thick gland structure (red arrowheads) **C:** Solid-type carcinoma, irregulary shaped lobuler structure (yellow arrowheads) **D:** Tubulopapillary-type carcinoma, papillary extensions (blue arrowhead) and tubular formation (blue arrow) **E:** Intraductular-type carcinoma, finger like projections (thin black arrow) supported by fibrous tissue **F:** Micropapillary-type carcinoma, irregular aggregates in tumour nodules (thin green arrows).

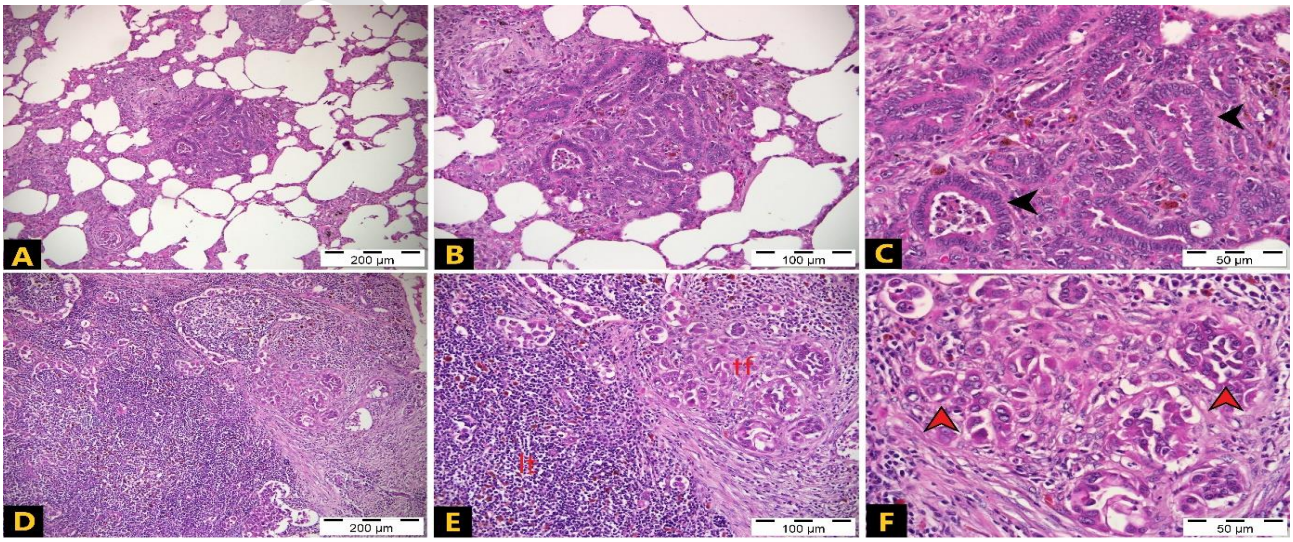

Fig. 3: Appearance of metastatic tumour lesions in the lung, tubular formations consist of neoplastic epithelial cells (black arrowheads) (A-B-C) and lymph node (lt), tumour foci (tf) and tumour cells (red arrowheads) (D-E-F) at different magnifications, H&E.

Immunohistochemical findings: All 20 cases were positive for BRCA1 and 2 immunoreactivity. BRCA1- and 2-positive labelling was primarily observed in the cytoplasm of epithelial tumour cells. No positive staining was found in the stromal cells, bone and cartilage components. BRCA1 and 2 expressions were found to be much more intense in early-grade cases compared to advanced-grade cases. In addition, no significant difference in BRCA1 and 2 immunoreactivity was observed between tumour subtypes (Fig. 4 & 5). Similar to the primary lesions, BRCA1 and BRCA2-positive reactions were observed in the cytoplasm of tumour cells within metastatic foci in the lymph nodes and lungs (Fig. 6). According to the malignancy grade, there was a statistically significant difference regarding BRCA1 and BRCA2 immunoreactivity between Grade 1 and 3 tumours ($P<0.01$). Significant differences in BRCA1 and BRCA2 immunoreactivity were observed between the metastatic and non-metastatic groups (Fig. 7).

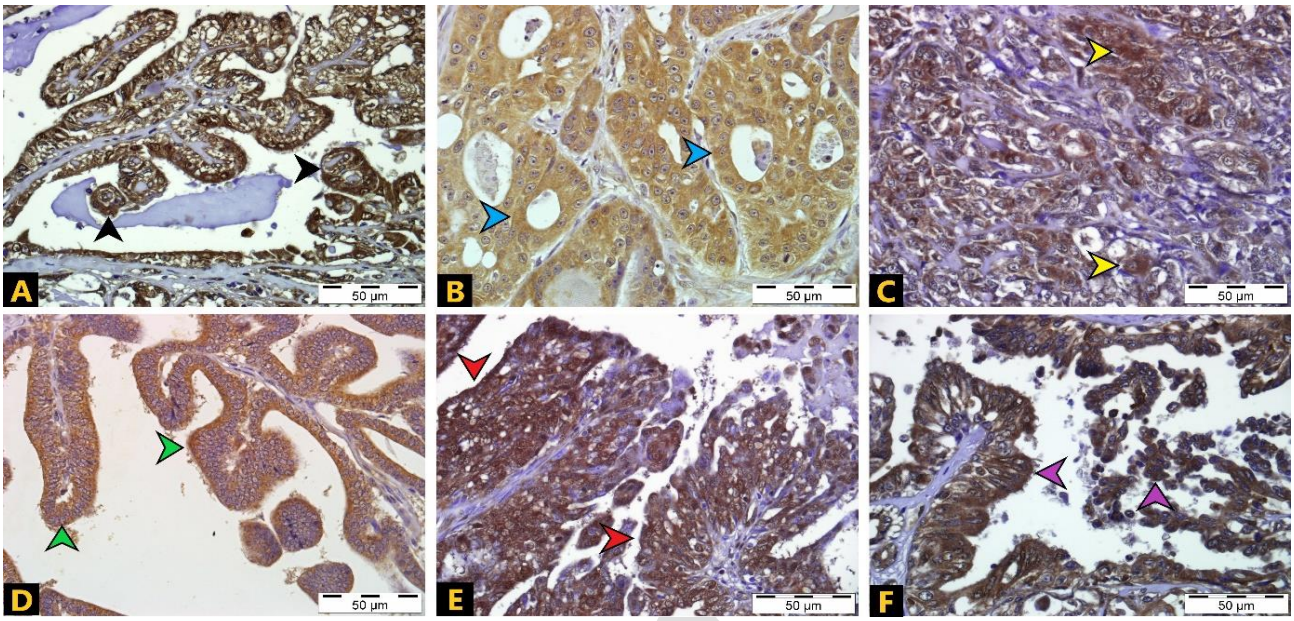

Fig. 4: BRCA 1, IHC, **A:** Mixed-type carcinoma, intracytoplasmic positive staining in the malignant epithelial component surrounding osteoid formations (black arrowheads) **B:** Tubular-type carcinoma, positive expressions in the cytoplasm of tumour cells forming glandular structures (blue arrowheads) **C:** Solid-type carcinoma, intracytoplasmic and quite severe dark brown immunoreactivities (yellow arrowheads) **D:** Tubulopapillary-type carcinoma, immunopositive reactions in the cytoplasm of malignant epithelial cells forming finger-like extensions (green arrowheads) **E:** Intraductular-type carcinoma, highly dense intracytoplasmic immune labellings in papillary structures (red arrowheads) **F:** Micropapillary-type carcinoma, cytoplasmic positive labelling in aggregates (purple arrowheads) within tumour nodule structures, Bar= 50 microns.

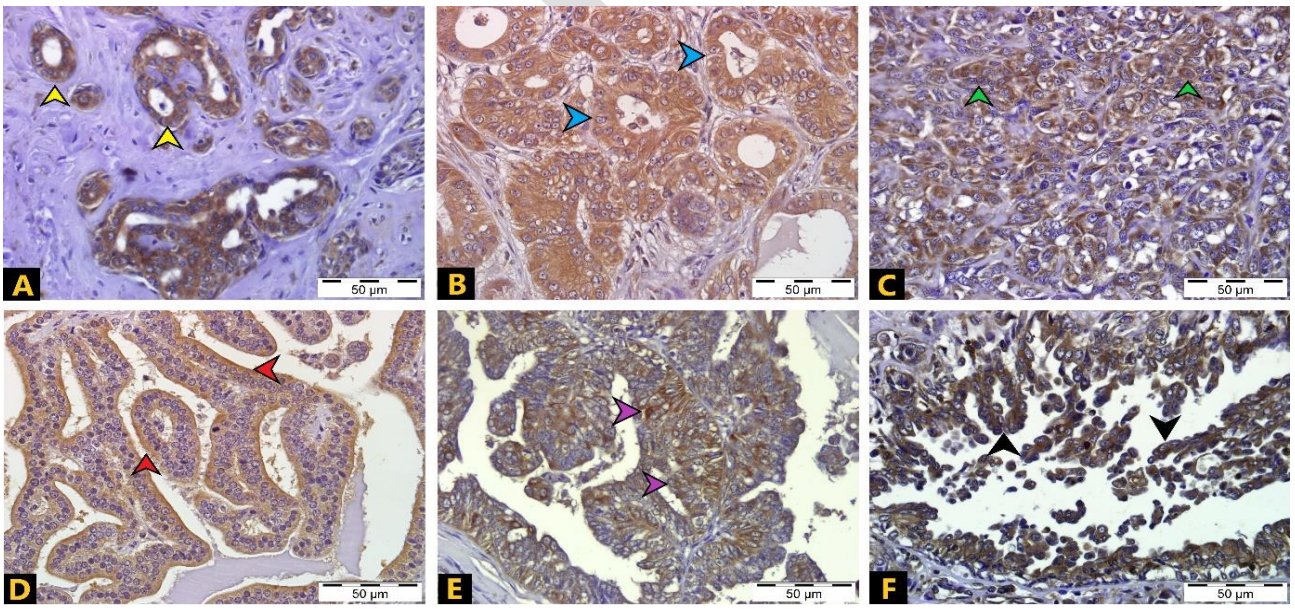

Fig. 5: BRCA 2, IHC, **A:** Mixed-type carcinoma, negative reaction in osteoid structures, positive labellings in the cytoplasm of epithelial tumour cells (yellow arrowheads) **B:** Tubular-type carcinoma, intracytoplasmic positive reactions in tubular structures formed by neoplastic cells (blue arrowheads) **C:** Solid-type carcinoma, intracytoplasmic positive reactions in cells arranged in arrays without a lumen (green arrowheads) **D:** Tubulopapillary-type carcinoma, immunepositive expressions in tubular and papillar formations (red arrowheads) **E:** Intraductular-type carcinoma, dark brown immunelabellings in the cytoplasm of neoplastic cells (purple arrows) **F:** Micropapillary-type carcinoma, immunopositive reactions in tumour cell masses (black arrowheads), Bar=50 microns.

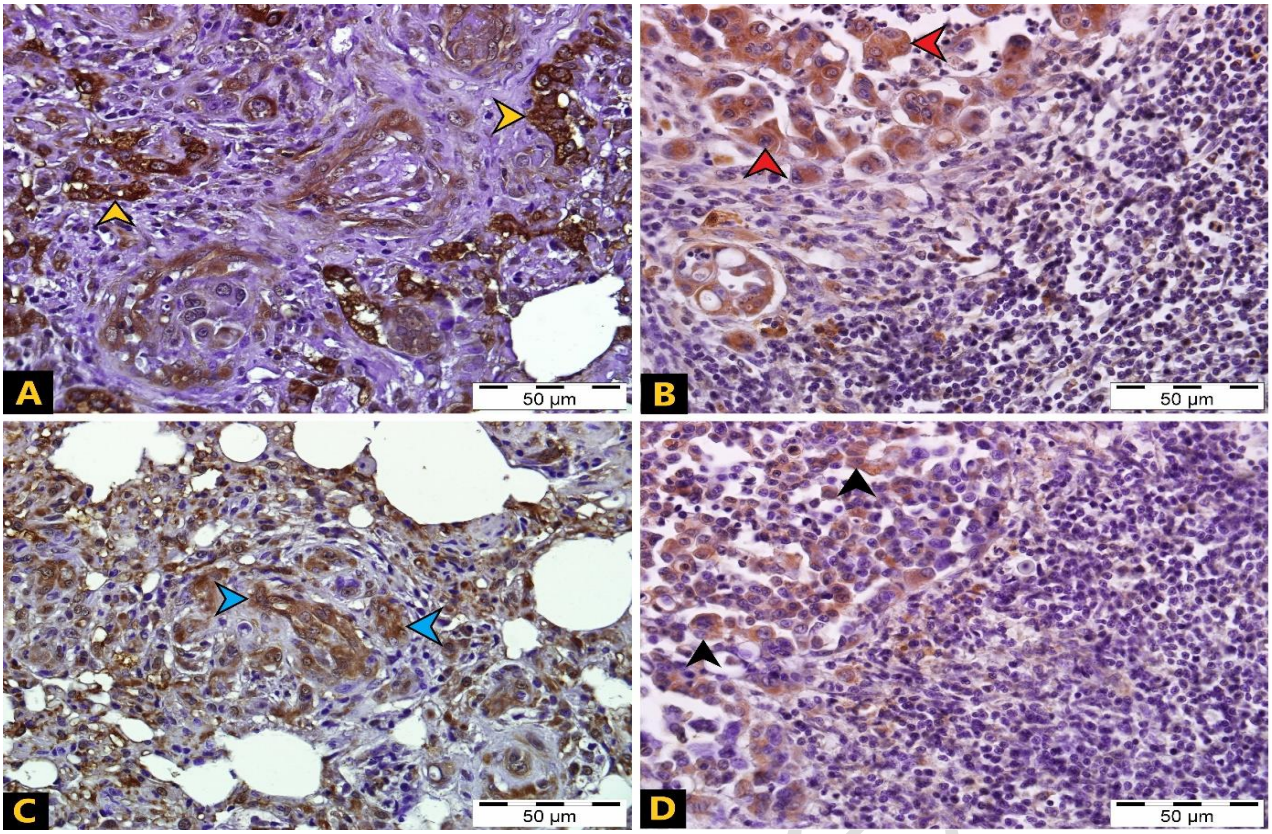

Fig. 6: BRCA1 (yellow arrowheads) and BRCA2 (blue arrowheads) immunoreactivity in metastatic foci within the lung, dark brown intracytoplasmic positive reactions in the malignant epithelial component **(A-C)**, and BRCA1 (red arrowheads) and BRCA2 (black arrowheads) immunopositive markings in the cytoplasm of tumour cells surrounding lymphoid tissue, lymph nodes **(B-D).**

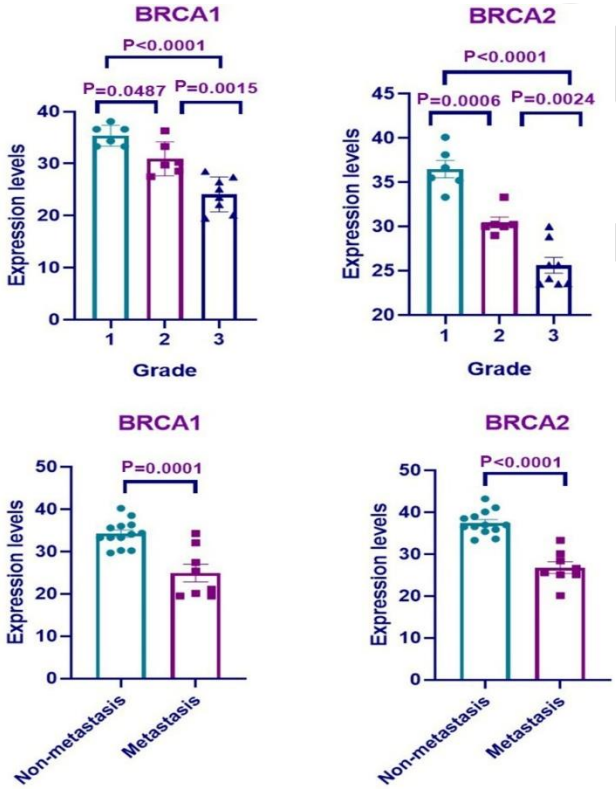

Fig. 7: BRCA1 and BRCA2 immunoreactivities in relation to grade and metastasis. A parametric two-group comparison (t-test) was performed to evaluate the expression levels of BRCA1 and BRCA2 between the metastatic and non-metastatic groups. A parametric one-way ANOVA followed by Tukey's post hoc test was employed to evaluate BRCA1 and BRCA2 expression values in Grade 1, 2, and 3 groups. Data were presented as mean±SEM.

DISCUSSION

Mammary gland tumours are common in both middle-aged and elderly dogs (Zuo *et al.*, 2024). In this study, the average age was 9.15 yaers, with most dogs falling within this range. Although Miniature Poodles, Malteses, Dachshunds, Yorkshire Terriers, German Shepherds and Cocker Spaniels are prone to mammary tumours, the predisposition to this cancer is much higher in mixed breeds (Namagerdi *et al.*, 2020). Consistent with the existing literature, the majority of cancer cases were observed in mixed breeds (9 of 20 cases, 45%).

Tumours often provide clues to their benign or malignant nature based on their external appearance. It has been suggested that smaller tumours are more benign than larger ones (Sorenmo *et al.,* 2009). Malignant tumours have indistinct borders, an inflamed appearance, are adherent to the overlying skin, and are usually ulcerated (Goldschmidt *et al.,* 2017). In the current study, all 20 cases were initially diagnosed as malignant based on preliminary macroscopic findings. However, when histopathological examinations were compared with these preliminary macroscopic diagnoses, the results were relatively consistent.

These tumours have a very poor prognosis (two-year survival rate of 25 to 40%) due to metastasis to the lungs, lymph nodes, spleen and liver, causing high mortality (Senhorello *et al.*, 2019; Yang *et al.*, 2023). In this study, distant tissue metastasis was found in 7 of 20 cases. The lung was identified as the most affected organ by metastasis.

Carcinoma in situ, carcinoma simple (tubular, tubulopapillary, cystic-papillary, cribriform),

micropapillary invasive carcinoma, solid carcinoma, comedocarcinoma, anaplastic carcinoma, complex carcinoma, carcinoma and myoepithelioma, malignant myoepithelioma, mixed carcinoma, ductal carcinoma, and intraductal papillary carcinomas are among the most common histological subtypes of malignant epithelial tumours of the mammary gland (Goldschmidt *et al.*, 2017). Although the sample size in this study was small, mixed-type, tubular-type, and solid-type carcinomas were quite predominant. Histological subtypes of canine mammary gland adenocarcinoma are considered an effective option for determining tumour behaviour (Munday *et al.*, 2019). Among metastatic cases, the dominant histological subtype was the solid type. In addition, among non-metastatic cases, the most common histological subtype was the mixed type. Half of the advanced-grade (3) cases were of the solid histological subtype, whereas there was a relative distribution among early-grade (1&2) cases. However, in the current study, no significant differences were observed between histological subtypes in terms of BRCA 1and 2 immunoreactivity.

Tumour markers are substances synthesised by either tumour cells or by the host organism at high concentrations in response to tumours. They are found in cells, tissues, blood, and body fluids, primarily aiding in auxiliary diagnosis, prognosis, evaluation of treatment effectiveness, and detecting disease relapse. They can serve as tumour indicators in early clinical diagnosis (Yang *et al.*, 2024). Early tumour detection, particularly malignant tumours, is a key milestone in modern cancer treatment. It enables more effective treatment and, thus, improved patient prognosis (Galadima *et al.*, 2024). Owing to the physiological, anatomical, and pathological similarities shared by human breast cancer and canine mammary tumours, biomarkers studied in canine mammary tumours may also provide insights into human breast cancer research (Yang *et al.*, 2023).

Currently, the examination of these tumour-related gene expression patterns has become essential for diagnosing and predicting outcomes in canine mammary gland tumours, especially for determining treatment. Although many of these biomarkers have been extensively studied in human breast cancer, studies focusing explicitly on canine mammary gland cancers are relatively few (Galadima *et al.*, 2024). In this study, the diagnostic efficacy of BRCA1 and BRCA2 expression in metastatic/non-metastatic canine mammary gland adenocarcinomas of different histological subtypes and grades was evaluated using immunohistochemistry.

The amino acid residues of the BRCA genes in humans and dogs are more than 80% similar (Yoshikawa *et al.*, 2021; Di Giacomo *et al.*, 2022). Chromosomal instability from BRCA gene inactivation is a major cause of carcinogenesis in the mammary gland, as it disrupts DNA repair and cytokinesis regulation (Gentile *et al.*, 2017). In women, inherited mutations in BRCA1 and BRCA2 account for approximately 10% of all breast cancer cases (Hsu *et al.*, 2010; de Oliveira *et al.*, 2022). Therefore, carriers of deleterious mutations (80%) face a high risk of developing the disease during their lives (Ozmen *et al.*, 2017; Arendt *et al.*, 2023). BRCA1 is an important tumour suppressor gene, involved in DNA repair, recombination, cell cycle checkpoints, transcription regulation, and triggering apoptosis in human breast cancer (Im *et al.*, 2013; Qiu *et al.*, 2015). Mutations in the BRCA1 gene reduce BRCA1 expression, thereby rendering BRCA1 dysfunctional and causing tumourigenesis (Qiu *et al.*, 2015; Qiu and Lin, 2016; Kwon *et al.*, 2023). BRCA2 is an important tumour suppressor gene. Mutations in this gene increase the risk of breast cancer in humans (Thumser-Henner *et al.*, 2020; Zhu *et al.*, 2023). Defects in BRCA2 prevent it from regulating cell development and proliferation, leading to carcinogenesis (Maués *et al.*, 2018).

The inactivation of tumour suppressor genes BRCA1 and BRCA2 due to the loss of heterozygosity elevates breast cancer risk in women by 56-87%. Moreover, somatic mutations in BRCA2 are linked to lymph node metastasis in humans (Gentile *et al.*, 2017). BRCA2 is an important tumour suppressor gene involved in double-strand DNA damage repair, thus maintaining genome stability (Maués *et al.*, 2018; Thumser-Henner *et al.*, 2020; Pasaol *et al.*, 2025). Few studies have evaluated the relationship between BRCA genes and canine mammary tumours (Ozmen *et al.*, 2017; Di Giacomo *et al.*, 2022;). However, considering the scarcity of studies focusing on canine BRCA2, a direct relationship between BRCA2 mutations and cancer risk has not yet been established (Zhu *et al.*, 2023). BRCA1 is closely associated with malignant tumours (Rivera *et al.*, 2009). Conversely, various researchers have found that BRCA1 and BRCA2 expression is reduced in canine mammary tumours compared to normal mammary tissue (Varney *et al.*, 2023; Yang *et al.*, 2023). It has been reported that decreased BRCA2 expression and mutations initiate mammary tumourigenesis in dogs, promoting cancer development (Maués *et al.*, 2018; Thumser-Henner *et al.*, 2020; de Oliveria *et al.*, 2022; Zhu et *al.*, 2023). In addition, studies have indicated that BRCA2 is associated with less aggressive tumour subtypes, better prognosis, and lower-grade carcinomas (Canadas-Sousa *et al.*, 2019). BRCA1 is a nuclear protein, and its functional loss leads to an abnormal distribution of BRCA1 in the cytoplasm in human ovarian and breast cancers (Nieto *et al.*, 2003; Im *et al.*, 2013). In this study, both BRCA1- and BRCA2-positive labelling were predominantly seen in the cytoplasm of tumour cells, a pattern frequently reported in the literature and considered to reflect mislocalisation rather than normal physiological distribution. Qiu *et al.* (2015) demonstrated that BRCA1 expression was significantly more intense in healthy control dogs than in those with malignant and benign mammary tumours. However, no significant difference in BRCA1 expression was reported between benign and malignant cases. On the other hand, existing literature indicates that decreased BRCA1 expression is associated with poor differentiation and increased proliferative activity (Nieto *et al.*, 2003; Gentile *et al.*, 2017). Yoshikawa *et al.* (2015) found a significant decrease in BRCA2 gene expression in mammary tumour cases, similar to the results observed in the BRCA1 gene. The current study found that both BRCA1 and BRCA2 expressions were decreased in advanced-grade cases compared to early-grade cases. However, immunohistochemical labelling revealed that the levels of this tumour suppressor gene were lower in metastatic cases compared to non-metastatic cases. Nieto *et al.* (2003) found that BRCA1 and BRCA2 were expressed

in 50% of lymph node metastases. Similarly, Klopfleisch and Gruber (2009) also noted that BRCA1 and BRCA2 were overexpressed in lymph node metastases. Consistent with these two studies, the current study found increased BRCA1- and BRCA2-positive labelling not only in lymph nodes but also in distant tissues, such as the lung, similar to the expression patterns observed in primary lesions. In light of these findings, decreased expression of BRCA1 and BRCA2 appears to contribute to more aggressive tumour behaviour and cancer development. BRCA1 and 2 appear to be significant biomarkers in the diagnosis of canine mammary tumours, the determination of treatment options, and the determination of prognosis. It is noteworthy that BRCA1 and BRCA2 are predominantly overexpressed in low-grade and non-metastatic cases, and also exhibit positive immunoreactivity in distant organs, such as the lungs and lymph nodes. This difference in immunohistochemical data was attributed to the use of mammary gland adenocarcinoma tissues from dogs of different age groups and breeds in the current study. However, it was concluded that the possibility of mutations in the BRCA1 and BRCA2 tumour suppressor genes at different levels in each case might also have caused these inconsistent findings.

Conclusions: Although no ideal biomarker for canine mammary gland tumours has yet been identified, the immunohistochemical data obtained from the current study statistically demonstrate that BRCA1 and BRCA2 are remarkable and useful biomarkers for determining the grade of malignancy, predicting prognosis, and revealing the metastatic capacity of canine mammary cancers.

Authors' contribution: EK: Designing the study, MK, CK, SK, MD: Surgical operations and data collection, EK, EB: Immunohistochemical and histopathological analysis, EK, AY: Immunohistochemical and histopathological staining, EK, MK: Data interpretation, data analysis, and statistical analysis, EK: Writing-Original drafting. All authors contributed to the manuscript and approved the final version for submission.

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