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RESEARCH ARTICLE

Investigation of Estrogen Receptor beta (ER-β), Progesterone Receptor (PR), Human Epidermal Growth Factor Receptor 2 (HER2), Programmed Death-Ligand 1 (PD-L1) and Cytotoxic T-Lymphocyte Associated Protein 4 (CTLA-4) Expression Across Histopathological Subtypes of Canine Mammary Masses

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ABSTRACT

Immunohistochemical findings in canine mammary masses (n=62) for estrogen receptor beta (ER-β), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), programmed death-ligand 1 (PD-L1), and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) are presented in this study. Expression of CTLA-4 differed between malignant and non-malignant masses ($P<0.05$), while HER2 expression differed between Grade II and Grade III tumors ($P<0.05$). Among the malignant mammary tumors, triple-negative mammary tumors (TNBC) accounted for 8.2%. Their PD-L1 expression scores were +2.5 in three cases and absent in one case. An association was found between PD-L1 expression and neutrophil-to-lymphocyte ratio ($P<0.001$). Our results revealed that HER2 expression may have prognostic value in high-grade tumors. Further investigation of immune checkpoint pathways in canine TNBC is needed for future treatment plans in female dogs with mammary tumor.

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INTRODUCTION

Mammary gland tumors are the most common neoplastic condition in intact female dogs (Sorenmo, 2003; Miao *et al.*, 2025). These tumors may be benign or malignant, and their development has been associated with age, breed, spaying status, diet, and environmental influences (Ariyarathna *et al.*, 2020; Kuruoglu *et al.*, 2020; Miao *et al.*, 2025). Histopathologic evaluation of canine mammary tumors is important for determining treatment strategies. Mastectomy is commonly performed in practice, and medical treatment approaches may also be considered (Franz *et al.*, 2021; Porcellato *et al.*, 2021; Mehrez *et al.*, 2025).

Surgical treatment of mammary gland tumors alone cannot cure metastatic disease. Some biomarkers can support accurate prognosis and guide therapeutic decisions. Estrogen receptor beta (ER-β), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), programmed death-ligand 1 (PD-L1), and cytotoxic T-lymphocyte–associated protein 4 (CTLA-4) are biomarkers currently under investigation in canine mammary tumors (Araújo *et al.*, 2016; Beckers *et al.*, 2016; Kim *et al.*, 2016).

Triple-negative mammary tumors (TNBC) are among the most aggressive breast cancer subtypes and are characterized by the absence of ER, PR, and HER2 expression, as well as a limited response to standard treatments. In human TNBC cases, antibodies targeting PD-1/PD-L1 and CTLA-4 have shown improved clinical outcomes (Maekawa *et al.*, 2014; Yang *et al.*, 2021).

In the present study, the immunohistochemical expression of ER-β, PR, HER-2 and PD-L1, CTLA-4 in mammary gland masses were evaluated in female dogs. The aims of the study are; (1) PD-L1 and CTLA-4 expression in TNBC, (2) the relation between the biomarkers and histopathological tumor grade, and (3) their association with preoperative leukocyte count (WBC), neutrophils (NEU), lymphocytes (LYM), monocytes (MON), eosinophils (EOS), basophils (BAS), neutrophil-to-lymphocyte ratio (NLR), tumor size, and postoperative survival.

MATERIALS AND METHODS

Cohort Selection and Postoperative Survival Evaluation: Canine mastectomy materials were collected between 2018

and 2022, and additional mammary gland samples were included from a biopsy archive in Türkiye. Sixty-two cases with clinical information (pre-surgical findings, reproductive status, and treatment history) were included in the study. The female dogs in the study were between 3 and 20 years old. Female dogs with pulmonary metastasis, abnormal regional lymph node involvement, or a history of treatments that could influence tumor behavior were excluded from the study. The post-mastectomy follow-up period (24 months) was conducted through owner communication or clinical visits, and overall survival (OS) was defined as the interval from mastectomy to death.

Hematological Analysis: A complete blood count was performed before the mastectomy and data were obtained using the ProCyte Dx Hematology Analyzer (IDEXX Laboratories, Westbrook, ME, USA). Evaluated parameters included WBC, NEU, LYM, MON, EOS, BAS, and NLR. The NLR was calculated by dividing neutrophil counts (10^9/L) to lymphocyte counts (10^9/L).

Histopathological Classification: Following gross examination, mammary tissue samples were processed using routine histological techniques and stained with hematoxylin and eosin for microscopy. Classification and grading of mammary tumors were carried out in accordance with the criteria described by Goldschmidt *et al.* (2011). Cases were categorized as malignant tumors (Group I, n=49), benign tumors (Group II, n=4), or hyperplasia/dysplasia (Group III, n=9). Malignant tumors were graded (I–III) using the modified Elston–Ellis's scoring system. All cases underwent immunohistochemical analysis. Macroscopic tumor measurements were available in only 29 cases in Group I.

Immunohistochemistry (IHC): For immunohistochemical analysis, 5µm sections prepared from tissues were placed onto adhesive microscope slides. Immunolabeling for PD-L1, HER2, CTLA-4, ER-β, and PR was performed using the streptavidin-biotin method. Positive control tissues included canine uterus, spleen, lymph node, skin, and mammary carcinoma samples. To obtain negative controls, an antibody diluent was used instead of the primary antibody.

Primary antibodies included PD-L1 (ABclonal, A1645, 1:300, incubation in a humid container at 37°C for 90 minutes), ER-β (Santa Cruz, SC-373853, 1:35, incubation overnight at 4°C in a humid environment container), PR (Santa Cruz, SC-810, 1:20, incubation overnight at 4°C in a humid environment container), HER2 (ABclonal, A2071, 1:150, incubation in a humid container at 37°C for 90 minutes), and CTLA-4 (Santa Cruz, SC-376016, 1:500, incubation in a humid container at 37°C for 90 minutes). For immunohistochemistry UltraVision Large Volume Detection System Anti-Rabbit, HRP (Ready-to-Use; Thermo Scientific, Waltham, MA, USA; Cat. No. TR-125-HL) visualized with 3,3′-diaminobenzidine (DAB) chromogen (Thermo Scientific, USA; Cat. No. TA-125-HD), and counterstained with Mayer's hematoxylin. EDTA buffer (pH 6.0) at 95°C was used for antigen retrieval.

Immunostaining Scoring: Immunostaining was evaluated semi-quantitatively based on staining extent and intensity in high-power fields (400×). At least 10 images from areas within mammary masses were assessed. Positive immunostaining for PD-L1, HER2, CTLA-4, ER-β, and PR was defined as a brownish color. The proportion of positively stained cells was scored as 0 (0–10%), +1 (10–30%), +2 (31–60%), or +3 (>60%). Samples showing no reactivity in the normal localization of the antibody were categorized as negative (score 0). Samples with ≥10% positive cells were considered positive, and intensity was graded as weak (+1), moderate (+2), or strong (+3). Scores were calculated as the arithmetic means of the extent and intensity scores.

Expressions of PD-L1 and CTLA-4 showed intracytoplasmic staining in epithelial and inflammatory cells. HER2 expression was assessed based on membranous immunostaining in mammary epithelial cells, whereas ER-β and PR demonstrated predominantly intranuclear staining, and only nuclear staining was scored. TNBC was defined by the absence of ER, PR, and HER2 expression.

Statistical Analysis: All statistical analyses were performed using SPSS software (version 29.0; IBM Corp., Armonk, NY, USA). Biomarker differences among study groups were analyzed using one-way ANOVA or Kruskal-Wallis tests with Tukey's HSD or Games-Howell post-hoc comparisons. Associations between biomarkers (PD-L1, CTLA-4, ER, PR, and HER2) and tumor size were assessed using Pearson's correlation test. Differences in biomarker expression among malignant tumor grades were evaluated using Kruskal-Walli's analysis. Relationships between hematological parameters (NEU, LYM, MON, EOS, BAS, NLR) and PD-L1 or CTLA-4 scores were also analyzed. Pearson's correlation test was used for correlations between the evaluated parameters and postoperative survival. Statistical significance was defined as $P<0.05$.

RESULTS

The mean age at diagnosis in female dogs was 10.1±2.5 years, 10.2±0.44 years in Group I, 9.5±1.19 years in Group II, and 9.11± 0.9 years in Group III. Most female dogs were intact (83.9%; n=52 dogs); among these, 41 were classified in Group I, 4 in Group II, and 7 in Group III. Breed distribution showed that small- and medium-sized breeds were more commonly affected. Mixed-breed dogs, Cocker Spaniels, and Golden Retrievers were mostly seen breeds in malignant mammary tumor's group (Supplementary Table 1).

Histopathological Findings: Histopathological evaluation of the 62 female dogs revealed malignant mammary tumors in 49 cases (Group I), benign tumors in 4 cases (Group II), and mammary hyperplasia/dysplasia in 9 cases (Group III). Group I tumors were further graded according to the modified Elston–Ellis's system: Grade I (n=7), Grade II (n=26), and Grade III (n=16). Malignant epithelial tumors were also grouped according to histological patterns, with predominant tumors including 12 complex carcinomas, 10 carcinosarcomas, 6 simple carcinomas, 5 complex adenoma/carcinoma mixes, 1 adenosquamous carcinoma, and 1 inflammatory carcinoma; the last two were classified as special types of malignant epithelial tumors. Benign tumors were as follows: 2 adenomas, 1 complex adenoma, and 1 benign mixed tumor. Hyperplasia/dysplasia group included 5 lobular hyperplasia, 2 ductal ectasias, and 2 fibroadenomatous differentiation. The distribution of the

mammary masses according to their histopathological classification is shown in Supplementary Table 4. TNBC cases were identified as 6.5% in all canine mammary masses and 8.2% in canine malignant tumors (Group I). TNBCs were classified as high-grade tumors (Grade III).

Of the 49 malignant tumors in group I, 46 showed a highly pleomorphic cell population, and polygonal cells were a prominent feature of all neoplasms. The tumors showed infiltrates of lymphocytes, plasma cells, and neutrophils. In group I, 57.1% (28/49) of tumors consisted of highly cellular areas. A high mitotic rate was identified at 400× in 61.22% (30/49) of cases. Focal necrotic areas were seen in 25 cases.

In Group I, four mammary tumors were triple-negative (ER−, PR−, HER2−). Histopathology of these TNBC tumors were 2 solid carcinomas grade III, 1 solid adenocarcinoma grade III, and 1 comedocarcinoma grade III.

Immunohistochemical Staining Scores: The mean values, and statistical significance levels of PD-L1, CTLA-4, HER2, PR, and ER-β immunohistochemical scores across the study groups are presented in Supplementary Table 5. Among the evaluated biomarkers, expression of CTLA-4 showed significancy between Group I and Group II, as well as between Group I and Group III and higher in Group I (P<0.001).

Group I was subdivided into Grade I (n=7), Grade II (n=26), and Grade III (n=16) with high mitotic index based on histopathological evaluation. The mean scores of PD-L1, CTLA-4, HER2, PR, and ER-β biomarkers according to histopathological grade subgroups are presented as Supplementary Table 6. HER2 expression was lower in Grade III tumors in comparison with Grade II (P<0.05). Expressions of PD-L1, ER-β, and PR were not differed between the study groups (P>0.05). Representative HE and IHC images for each group are shown in Fig. 1-3.

Three of TNBC cases showed strong immunoreactivity of PD-L1 (+2.5) and CTLA-4 (+2.5, +2), whereas one case showed completely negative (0) staining for PD-L1 and low (+1) staining for CTLA-4 (Table 1). Due to the limited number of TNBC cases, no statistical comparison was made for this subgroup. HE representative images for TNBC are shown in Fig. 4.

Relationship Between Preoperative Blood Count and Immune Marker Expression: There are no associations between PD-L1 and CTLA-4 expression with NEU, LYM, MON, EOS, or BAS (P>0.05). There is a significant difference in NLR values according to PD-L1 scores of +0, +1, +1.5, +2 and +3 (ANOVA/Kruskal-Wallis test, P<0.001). Correlation analysis of NLR values and PD-L1 scores showed that PD-L1 score of +2.5 was associated with a high NLR value of 5.28±0.46 (post-hoc comparisons). There is no association between NLR values and any of CTLA-4 scores (P>0.05). Blood cell parameters and their relationships with immune biomarker expression scores are presented in Supplementary Tables 2-3.

Table 1: PDL-1 and CTLA-4 biomarker scores of triple-negative canine mammary tumors.

Sample no&Breed	OVH	Histopathology	Survival time after mastectomy	PDL-1 Score	CTLA-4 Score
#1 Cocker Spaniel	+	Solid Carcinoma, Grade III	≥24 months*	2.5	2.5
#9 Golden Retriever	+	Solid Carcinoma, Grade III	6 months	2.5	2
#22 King Charles Spaniel	–	Solid Adenocarcinoma, Grade III	2 months	2.5	2
#29 Boxer	–	Comedocarcinoma, Grade III	≥24 months*	0	1

*The follow-up period after mastectomy surgery was 24 months.

Supplementary Table 1: Breed distribution of female dogs in each histopathological group

Breed	Group I (n=49)	Group II (n=4)	Group III (n=9)	Total (n=62)
German Shepherd Dog	2 (3.2%)	0	1 (1.6%)	3 (4.8%)
Akita Inu	1 (1.6%)	0	0	1 (1.6%)
Beagle	2 (3.2%)	0	0	2 (3.2%)
Boxer	2 (3.2%)	0	0	2 (3.2%)
Chihuahua	1 (1.6%)	0	0	1 (1.6%)
Cocker Spaniel	8 (12.9%)	0	3 (4.8%)	11 (17.7%)
Dogo Argentino	0	0	1 (1.6%)	1 (1.6%)
French Bulldog	1 (1.6%)	0	0	1 (1.6%)
Golden Retriever	7 (11.3%)	1 (1.6%)	0	8 (12.9%)
Jack Russel Terrier	0	1 (1.6%)	0	1 (1.6%)
King Charles Spaniel	2 (3.2%)	1 (1.6%)	0	3 (4.8%)
Anatolian Shepherd Dog	1 (1.6%)	0	0	1 (1.6%)
Labrador Retriever	1 (1.6%)	0	0	1 (1.6%)
Mongrel	9 (14.5%)	1 (1.6%)	1 (1.6%)	11 (17.7%)
Pinscher	1 (1.6%)	0	1 (1.6%)	2 (3.2%)
English Setter	1 (1.6%)	0	0	1 (1.6%)
Yorkshire Terrier	4 (6.5%)	0	0	4 (6.5%)
Terrier	6 (9.7%)	0	2 (3.2%)	8 (12.9%)

Tumor Macroscopic Size and Biomarker Expression: Among the 29 dogs with available measurements, the mean macroscopic tumor diameter was 1.79 ± 0.81 cm (mean±SD). The relationships between tumor macroscopic size and the expression scores of PD-L1, CTLA-4, HER2, PR, and ER-β were evaluated. Tumor macroscopic size showed negative correlations with PD-L1 and ER-β expression and positive correlations with CTLA-4, HER2, and PR expression (P>0.05).

Relationship between PD-L1, CTLA-4, HER2, PR, and ER-β Biomarkers and Postoperative Survival: The mean postoperative survival times for Groups I, II, and III were 17.79±8.57, 21.00±6.00, and 19.25±8.94 months (mean±SE), respectively. No statistical associations were found between biomarker expression and postoperative survival time in any group (P>0.05).

Supplementary Table 2: PD-L1 Scores vs Preoperative Blood Parameters

PD-L1 Score	n	LYM (×10³/µL)	MON (×10³/µL)	NLR	NEU (×10³/µL)	EOS (×10³/µL)	BAS (×10³/µL)
0	5	2.54±0.65	1.35±0.53	3.04±0.07^{b}	7.72 ±1.99	0.59±0.18	0.01±0.002
1.5	5	1.48±0.90	1.01±0.36	7.12±1.84^{ab}	10.22±2.54	0.68±0.28	0.07±0.03
2	14	3.35±0.86	1.47±0.26	4.32±0.41^{ab}	14.73±4.11	0.50±0.09	0.03±0.01
2.5	24	2.10±0.26	1.01 0.13	5.28±0.46^{a}	11.30±2.35	0.48±0.09	0.03±0.01
3	11	1.92±0.16	0.80±0.09	3.49±0.33^{b}	6.50±0.57	0.40±0.06	0.02±0.01
Reference Value	–	1.05–5.10	0.16–1.12	–	2.95–11.64	0.06–1.23	0–0.10
P-value	–	0.228	0.221	***0.001	0.413	0.732	0.258

Values were given as Mean±SD. N+number of cases; LYM, lymphocytes; MON, monocytes; NEU, neutrophils; EOS, eosinophils; BAS, basophils; NLR, neutrophil-to-lymphocyte ratio; SD, standard deviation. Notes: Regarding PD-L1 scores: 0 → 3 female dog Group I, 1 female dog Group II, 1 female dog Group III; 1.5 → 4 female dog Group I, 1 female dog Group III; 2 → 11 female dog Group I, 1 female dog Group II, 2 female dog Group III; 2.5 → 20 female dog Group I, 1 female dog Group II, 3 female dog Group III; 3 → 9 female dog Group I, 2 female dog Group III. Superscripts ($^{a-b}$) indicate statistically significant differences between groups (post-hoc test). ***; NLR values differentiated between the PD-L1 scores (ANOVA/Kruskal-Wallis's test, P<0.001).

Supplementary Table 3: CTLA-4 Scores vs Preoperative Blood Parameters

CTLA-4 Score	n	LYM (×10³/μL)	MON (×10³/μL)	NLR	NEU (×10³/μL)	EOS (×10³/μL)	BAS (×10³/μL)
0	7	1.77±0.39	0.88±0.35	4.04±0.32	6.75±1.08	0.50±0.14	0.01±0.009
1	5	1.69±0.13	1.21±0.08	5.05±0.93	8.17±1.03	0.81±0.25	0.05±0.04
1.5	15	3.05±0.82	1.35±0.26	4.66± 0.74	13.73±3.89	0.30±0.06	0.02±0.01
2	21	2.12±0.15	0.88 ±0.10	4.45± 0.43	9.06±1.04	0.56 ±0.09	0.02± 0.01
2.5	10	2.65±0.59	1.40±0.29	5.03± 0.80	14.42±5.43	0.50± 0.08	0.03± 0.01
3	2	1.56± 0.20	0.58±0.32	6.55± 2.76	9.72±3.03	0.31±0.21	0.02± 0.01
Reference Value	–	1.05–5.10	0.16–1.12	–	2.95–11.64	0.06–1.23	0–0.10
P-value	–	0.510	0.274	0.785	0.551	0.124	0.768

Values were given as Mean±SD. N+number of cases; LYM, lymphocytes; MON, monocytes; NEU, neutrophils; EOS, eosinophils; BAS, basophils; NLR, neutrophil-to-lymphocyte ratio; SD, standard deviation. Regarding CTLA-4 scores: 0 → 2 female dog Group I, 2 female dog Group II, 3 female dog Group III; 1 → 3 female dog Group I, 1 female dog Group II, 1 female dog Group III; 1.5 → 13 female dog Group I, 2 female dog Group III; 2 → 18 female dog Group I, 3 female dog Group III; 2.5 → 10 female dog Group I; 3 → 2 female dog Group I

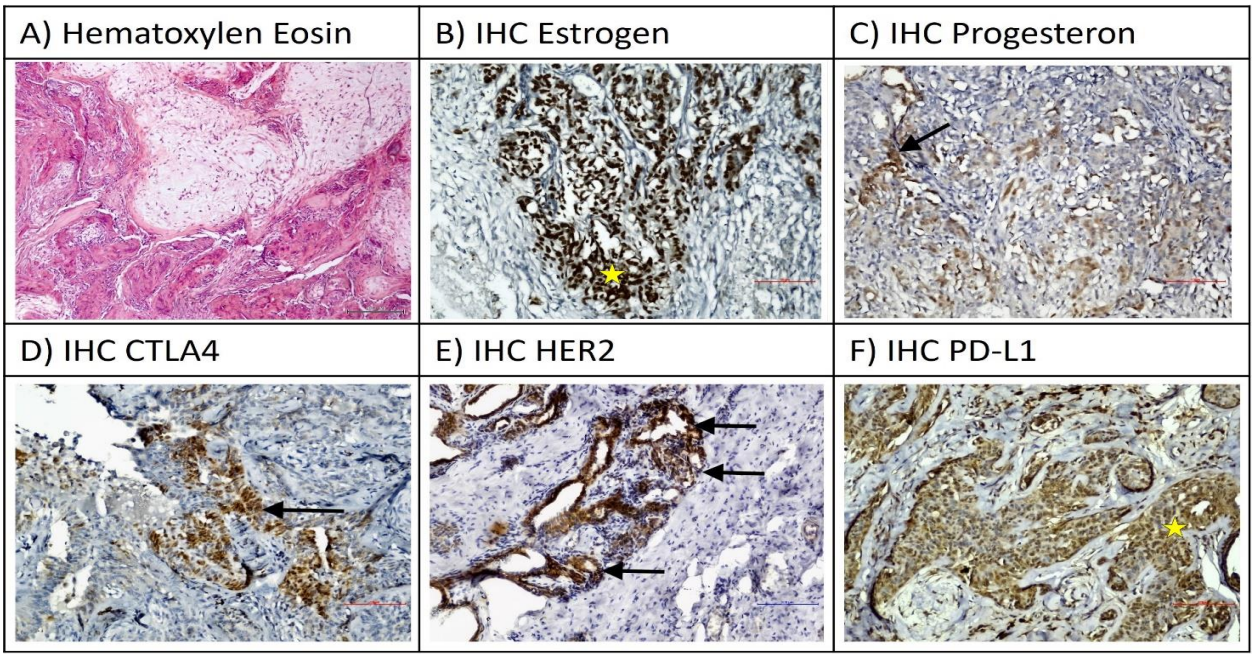


Fig. 1: Histological and immunohistochemical images of canine malignant mammary tumor (Group I). A) Histopathological appearance (H&E) B) Intense intranuclear immunostaining of ER-β in tumor cells C) Intranuclear immunopositivity of tumor cells with PR D) Intracytoplasmic immunopositivity of tumor cells with CTLA4 E) Membraneous and intracytoplasmic immunopositivity of tumor cells with HER2 F) Membraneous and intracellular staining of PD-L1 positivity in malignant mammary tumor cells. Ground Mayer's hematoxylin, chromogen DAB, Bar=90μm. Immunopositivity is shown as brownish color. Arrows indicate positive cytoplasmic and membraneous staining of cells. Yellow stars indicate positively stained areas.

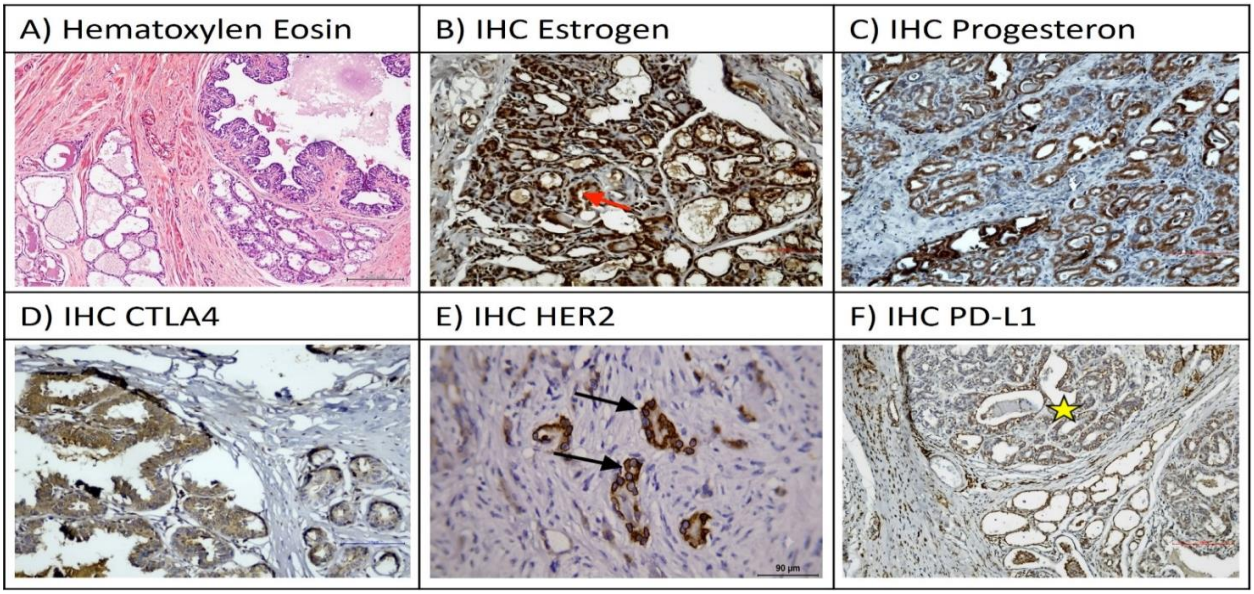


Fig. 2: Images of canine benign mammary tumor (Group II). A) Histopathological appearance (H&E) B) Intense intranuclear immunostaining of ER-β in tumor cells C) Intranuclear immunopositivity of tumor cells with PR D) Intracytoplasmic immunopositivity of tumor cells with CTLA4 E) Membraneous and intracytoplasmic immunopositivity of tumor cells with HER2 F) Membraneous and intracellular staining of PD-L1 in tumor cells. Ground Mayer's hematoxylin, chromogen DAB, Bar =90 μm. Immunopositivity is shown as brownish color. Red arrow indicates positive intranuclear staining of cells. Black arrows indicate positive cytoplasmic and membraneous staining of cells. Yellow star indicate positively stained areas.

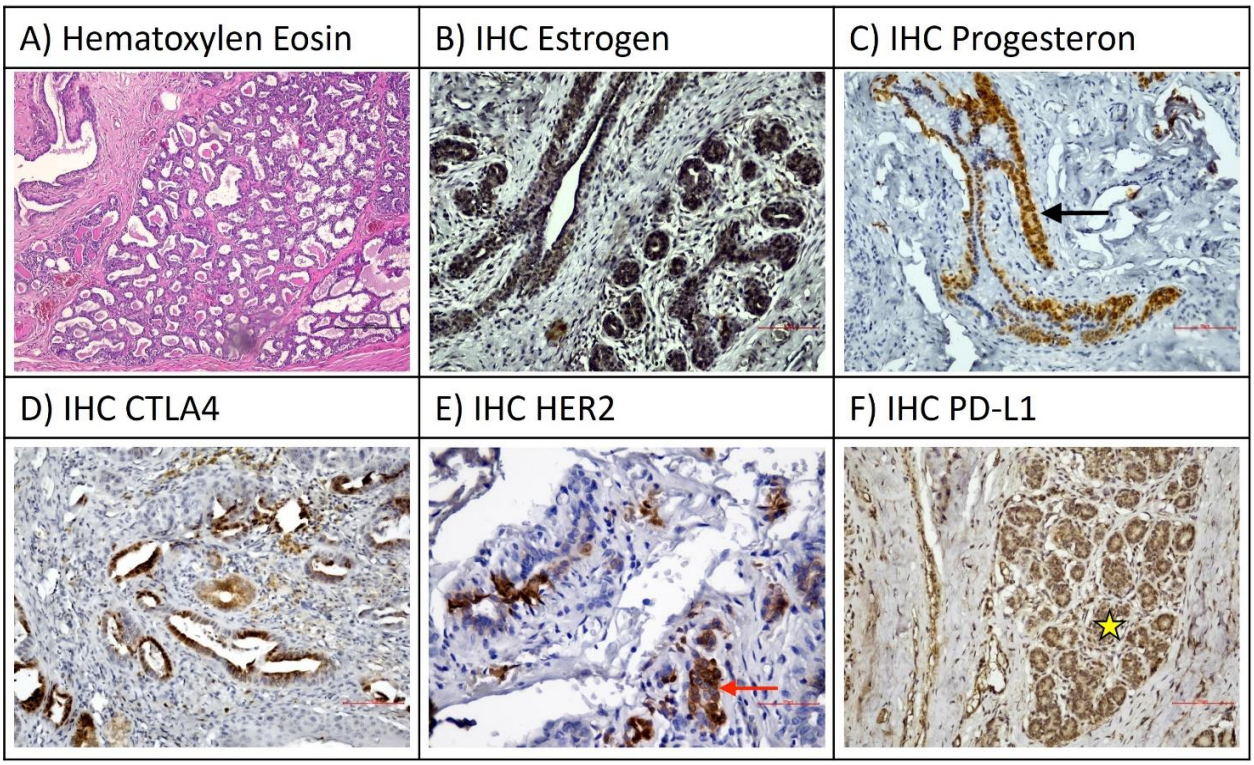


Fig. 3: Images of canine mammary hyperplasia (Group III). A) Histopathological appearance (H&E) B) Intranuclear immunostaining of ER-β in mammary cells C) Intranuclear immunopositivity of cells with PR D) Intracytoplasmic immunopositivity of cells with CTLA4 E) Membraneous and intracytoplasmic immunopositivity of cells with HER2 F) PD-L1 positivity. Intracellular staining in cells. Ground Mayer's hematoxylin, chromogen DAB, Bar =90µm. Immunopositivity is shown as brownish color. Black arrow indicates positive cytoplasmic and membraneous staining of cells. Red arrow indicates positive cytoplasmic and membraneous staining of cells. Yellow star indicate positively stained areas.

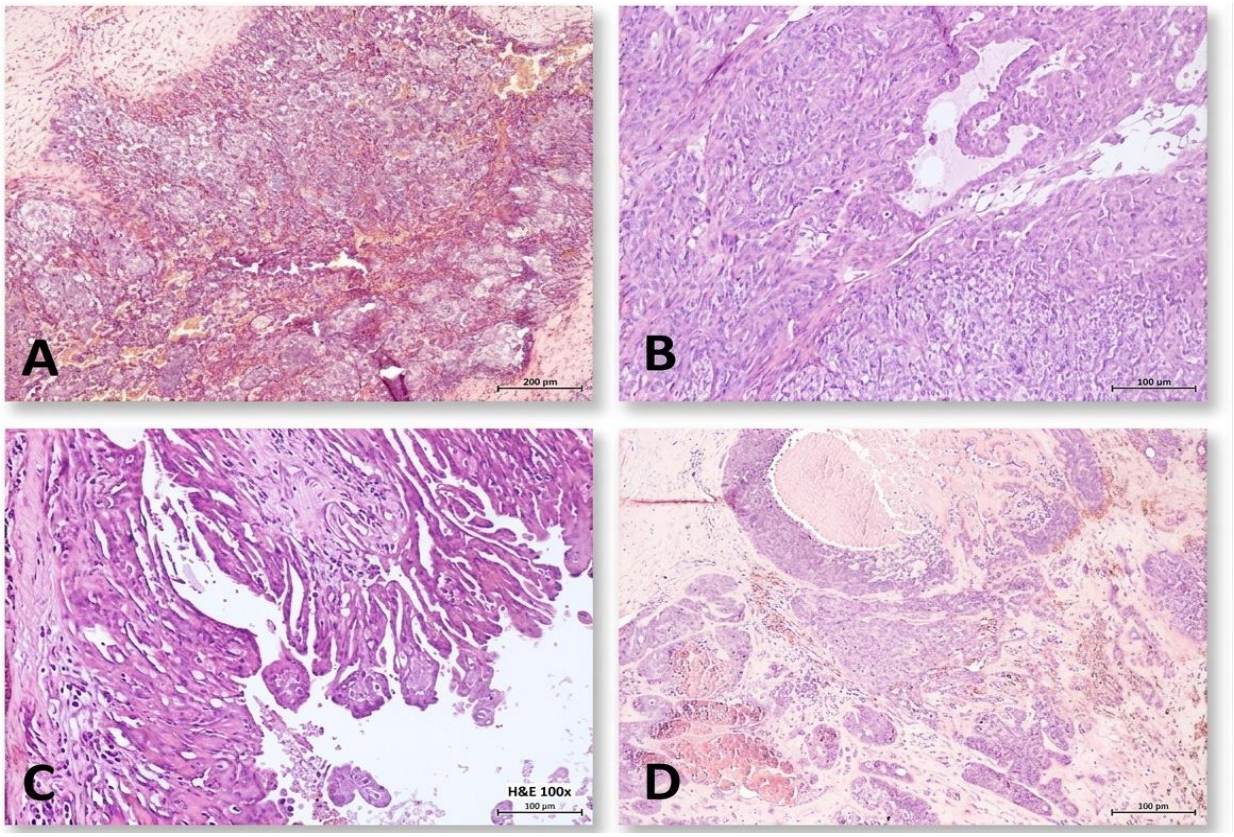


Fig. 4: Histopathologic spectrum of triple-negative canine mammary carcinoma (H&E stain) A) Solid invasive growth pattern: Loss of normal glandular architecture replaced by expansive, solid sheets of neoplastic epithelial cells infiltrating fibrous stroma (Bar: 200µm). B) High-grade cytological atypia: Severe cellular pleomorphism, hyperchromatic nuclei with prominent nucleoli, high nuclear-to-cytoplasmic ratios, and frequent mitotic figures (Bar: 100µm). C) Papillary architecture: Complex papillary to micropapillary projections lined by dysplastic, pleomorphic epithelial cells with focal luminal detachment and active stromal invasion (Bar: 100µm). D) Comedocarcinoma and necrosis: Characteristic central (comedo-type) necrosis with necrotic eosinophilic debris and focal hemorrhage surrounded by viable tumor cell nests (Bar: 200 µm).

Supplementary Table 4: Histopathological Classifications of Canine Mammary Masses

Histopathological Classification	n
Malignant Epithelial Tumors	
Carcinoma in situ	1
Simple carcinoma	6
Solid carcinoma	4
Comedocarcinoma	1
Complex adenoma/carcinoma mix	5
Complex carcinoma	12
Carcinoma and malignant myoepithelioma	2
Mixed carcinoma	2
Intraductal papillary carcinoma	3
Malignant Epithelial Tumors – Special Types	
Adenosquamous carcinoma	1
Inflammatory carcinoma	1
Malignant Mesenchymal Tumors (Sarcoma)	
Osteosarcoma	1
Carcinosarcomas – Mixed Malignant Tumors	10
Benign Tumors	
Adenoma	2
Complex adenoma	1
Benign mixed tumor	1
Hyperplasia / Dysplasia	
Ductal ectasia	2
Lobular hyperplasia (Adenosis)	5
Fibroadenomatous differentiation	2

In Group I, positive correlations were observed between post-mastectomy survival and the immunohistochemical scores of PD-L1, HER2, and ER-β, whereas negative correlations were observed for CTLA-4 and PR scores (P>0.05). In Group II, postoperative survival showed positive correlations with HER2 and ER-β scores, and negative correlations with PD-L1, CTLA-4, and PR scores (P>0.05). In Group III, a positive correlation was observed between survival and PR scores, and negative correlations with PD-L1, CTLA-4, HER2, and ER-β scores (P>0.05).

Supplementary Table 5: Immunohistochemical Scores of Biomarkers

Biomarker	Group I (n=49) Mean±SE	Group II (n=4) Mean± SE	Group III (n=9) Mean± SE	*P*- value
PD-L1	2.20±0.11	1.50±0.54	2.10±0.31	0.270
CTLA-4	1.85±0.09[a]	0.75±0.48[b]	1.10± 0.30[b]	***0.001
HER-2	1.35±0.13	1.25± 0.48	1.39± 0.22	0.982
PR	1.30± 0.12	1.00± 0.61	1.06± 0.28	0.682
ER-β	1.33± 0.16	1.50±0.54	1.67± 0.43	0.613

SE, standard error. Different superscript letters ([a], [b]) indicate statistically significant differences between groups (***P<0.001).

Supplementary Table 6: Mean scores of mammary tumors in Group I according to histopathological grade

Biomarker	Grade I (n=7) Mean ±SE	Grade II (n=26) Mean ±SE	Grade III (n=16) Mean±SE	P-value
PDL-1	2.21±0.391	2.25±0.128	2.13± 0.191	0.728
CTLA-4	1.79±0.184	1.90±0.100	1.78 ±0.209	0.867
HER-2	1.14± 0.237[b]	1.65±0.173[a]	0.94± 0.203[b]	*0.037
PR	1.21± 0.391	1.44±0.155	1.09±0.225	0.488
ER-β	2.00± 0.267	1.44± 0.217	0.84±0.291	0.084

SE, standard error. Different superscript letters indicate statistically significant differences between grades (*P<0.05).

DISCUSSION

Mammary tumors are among the most frequently observed neoplastic diseases in female dogs, and their development is influenced by factors such as breed, diet, advanced age, environment, spaying status, pseudopregnancy, and radiation exposure (Sorenmo, 2003; Ariyarathna *et al.,* 2020). We analyzed aging factor on mammary tumor development in our study and found an increased risk of malignant mammary tumor development with age in female dogs. Our findings are consistent with those of Zatloukal *et al.* (2005), who reported mean ages of 10, 8.9, and 8.3 years in the malignant tumor, benign tumor, and hyperplasia/dysplasia groups, respectively.

Tumor cells can exploit immune checkpoint pathways to reduce anti-tumor immune responses, and with this feature they facilitate their survival and progression. Immune checkpoint inhibitors restore this by activating T-cells and promoting anti-tumor immunity (Tang *et al.*, 2025). PD-L1 and PD-1 are among the best-known immune checkpoints and PD-L1 existence with increased percentages within tumor reduces immune recognition of tumor cells and suppress immune responses against cancer cells (Namagerdi *et al*., 2020; Giuliano *et al*., 2024). In veterinary oncology, PD-L1 expression has been investigated in tumors, and it has been shown to be present in certain cancers such as osteosarcoma, mast cell tumors, oral melanoma, hemangiosarcoma, and mammary carcinoma (Maekawa *et al*., 2014; Maekawa *et al*., 2016; Shosu *et al*., 2016; Hartley *et al*., 2017; Takeuchi *et al*., 2020; Ariyarathna *et al*., 2020). Increased percentages of PD-L1 are associated with shorter survival time in dogs with cancer (Ariyarathna *et al*., 2020). On the other hand, Lopes-Neto *et al*. (2021) reported no significant difference in survival between PD-L1 positive and PD-L1 negative canine mammary tumors. In our study, no statistically significant association was observed between post-mastectomy survival and PD-L1 expression in female dogs any of the groups (p>0.05).

In a canine study conducted by Maekawa *et al.* (2016), PD-L1 immunoreactivity was detected in four of five mammary adenocarcinomas. A later investigation by Shosu *et al.* (2016) demonstrated similar positivity rates in adenocarcinomas and adenomas, with PD-L1 expression observed in 66.7% of samples from each group. Lopes-Neto *et al.* (2021) detected PD-L1 expression in 39% of cases in their study on simple and complex mammary carcinomas. Other studies have reported a wide range of PD-L1 positivity rates in canine mammary carcinoma, ranging from 3% to 100% (Maekawa *et al*., 2021; Muscatello *et al*., 2024). In the present study, PD-L1 expression was detected in 83.7% of malignant tumors and 75% of benign tumors. Differences in positivity rates compared with previous studies are likely influenced by methodological factors such as antibody selection (Vlajnic *et al*., 2022), tumor heterogeneity, histopathological grade, and scoring criteria.

In our study, PD-L1 scores were observed higher in Group I (2.20±0.105) in comparison with Group II (1.50±0.540) and Group III (2.10±0.309) (P>0.05). There is similarity of PD-L1 scores with the previous studies (Shosu *et al*., 2016; Ariyarathna *et al*., 2020; Galadima *et al*., 2024). This situation may reflect the widespread expression of PD-L1 in the tumor microenvironment and its role in facilitating evasion of the immune system. The lack of significant differences between groups may be related to the presence of mixed histopathological subtypes, disparity in sample distribution, or the generally high frequency of PD-L1 positivity in both benign and malignant tumors, as noted in previous reports.

Triple-negative breast cancer accounts for approximately 15–20% of breast cancers in women (Namagerdi *et al*., 2020) and is associated with poor

prognosis, high probability of metastasis and short survival (Beckers *et al.*, 2016). A study on canine mammary tumors found that 18.7% of tumors were TNBC (Kim *et al*., 2013), another study reported a prevalence of 16.9% (Pastor *et al.*, 2020), and 14.1% (Razavirad *et al*., 2024). In our study, four dogs were found TNBC, representing 8.16% of Group I. A study on cats with TNBC showed significantly higher serum PD-1 and PD-L1 levels (Nascimento *et al*., 2020). In our study, three of the TNBC cases showed positive PD-L1 expression, and all four showed positive CTLA-4 expression. Immunohistochemical analysis performed with three different commercial antibody clones in women with TNBC showed PD-L1 expression positivity in 59.5%, 52.4%, and 34.5%, respectively (Vlajinic *et al*., 2022). Studies in human medicine have shown CTLA-4 expression positivity ranging from 36% to 82% (Navarrete-Bernal *et al*., 2020; Cabioglu *et al.*, 2021; Vardas *et al.*, 2023). PD-L1 immunomarker expression studies in TNBC are limited, and to our knowledge, there are no published studies yet for CTLA-4. Therefore, it is suggested that future studies investigating the expression of immune checkpoint inhibitors in a larger number of female dogs diagnosed with TNBC could guide the development of immunotherapeutic treatment methods for malignant canine mammary tumors.

T-cell activation is negatively regulated by CTLA-4, which has a key role in tumor immunity (Wolchok *et al.,* 2013; Topalian *et al.,* 2016). It is expressed on activated T cells and interacts with B7 ligands, thereby modulating T-cell responses. Inhibition of CTLA-4 enhances anti-tumor immune responses by increasing T-cell activity (Tang *et al*., 2025). Data about CTLA-4 expression in CD4+ and CD8+ T cells remains limited and most of the studies were done on histiocytic tumors, high-grade B-cell lymphoma, melanocytic tumors and mammary tumors in canine oncology (Pinard *et al*., 2022). Upregulation of CTLA-4 has been related to suppression of anti-tumor activity in human (Wolchok *et al*., 2013; Topalian *et al*., 2016). Comparable results have been observed in dogs, as CTLA-4 expression was more pronounced in malignant mammary lesions than in benign or hyperplastic mammary tissues (Pinard *et al.*, 2022).

Although some studies have suggested a possible association between elevated CTLA-4 expression and poorer prognosis, this relationship remains inconsistent across reports (Ariyarathna *et al*., 2020). In the present study, no significant association was observed between CTLA-4 expression and post-mastectomy survival among the groups ($P>0.05$). However, CTLA-4 expression showed significant differences between Groups I and II, as well as between Groups I and III ($P<0.05$). These findings indicate that CTLA-4 expression may be associated with tumor type in canine mammary masses and could have potential value in distinguishing malignant tumors from benign and hyperplastic/dysplastic lesions. Higher CTLA-4 expression observed in malignant tumors is in line with its reported role in suppressing anti-tumor immune responses within the tumor microenvironment (Marin-Acevedo *et al*., 2018). CTLA-4 has been shown to interfere with T-cell activation, thereby reducing immune-mediated recognition of tumor cells (Pardoll, 2012). The relatively lower expression observed in benign and hyperplastic tissues may reflect differences in immune regulation across tumor types rather than a clearly defined immune escape mechanism.

The association between PD-L1 and CTLA-4 expression and the immune response in canine mammary tumors provides important clues for immunotherapeutic strategies and increases the potential for predicting treatment response to immune checkpoint inhibitors. PD-L1 expression has been found to correlate strongly with hematological markers such as neutrophil-lymphocyte ratio (NLR), lymphocyte-neutrophil ratio (LNR), and platelet-neutrophil ratio (PNR) (Diaz *et al.*, 2019; Franz *et al.*, 2021). A high NLR has been reported to support PD-L1 positivity by creating a pro-inflammatory immune environment, and low LNR and PNR values also correlate with PD-L1 positivity (Nakamura *et al.*, 2019; Ekici *et al*., 2023). In particular, low LNR and PNR values have been reported to correlate with PD-L1 positivity, indicating that systemic inflammation plays a decisive role in the tumor immune microenvironment (Diaz *et al.*, 2019). A strong association between NLR and platelet-lymphocyte ratio (PLR) and immunotherapy response and overall survival has been emphasized (Rossi *et al.*, 2020; Franz *et al*., 2021). In our study, we found a correlation between a PD-L1 score of +2.5 and higher NLR values, and there was a significant difference in NLR values according to PD-L1 scores ($P<0.001$). This result is consistent with other studies (Nakamura *et al.*, 2019; Ekici *et al*., 2023).

Estrogen plays a fundamental role in mammary gland physiology and cell proliferation, and its receptor status has been recognized as an important factor in human breast cancer prognosis (Anderson, 2002; Diaz Bessone *et al*., 2019). In female dogs, ovarian hormones such as estrogen and progesterone, are also implicated in mammary tumor development (Torres *et al.*, 2021). The timing of ovariohysterectomy is considered a relevant factor in reducing the risk of mammary tumor formation (Benavente *et al.*, 2016). In our study, population of spayed female dogs were 16.12% and intact female dogs were 83.82%. In a study, ER-positive cases were compared with ER-negative ones, and longer survival was observed in ER-positive cases (Brunetti *et al*., 2021). In another study, ER expression was generally found to be reduced in malignant lesions (Peña *et al*., 2014). In this study, ER expression did not differ between the tumor groups or the hyperplastic mammary mass group, there was no association between ER expression and survival.

Previous studies have also described higher ER expression in lower-grade malignant tumors and in benign lesions compared with malignant tumors (de Las Mulas *et al.*, 2005; Brunetti *et al*., 2021). In addition, comparisons of ER subtypes have suggested an association between higher serum E2 levels and lower histological grades, supporting a possible role of estrogenic activity in tumor progression in ERα-positive mammary carcinomas (Sorenmo *et al*., 2019). Ekici *et al*. (2023) reported that NLR was significantly different between dogs with malignant mammary tumors and the control group. In our study, we evaluated the correlation between NLR and PD-L1 and found an association between high NLR (>5) and a high PD-L1 score (+2.5).

Conclusions: This study evaluated the expression of PD-L1, CTLA-4, ER-β, PR, and HER2 in canine mammary masses, and the main findings can be summarized as follows:

1. **CTLA-4** distinguished malignant from benign and hyperplastic/dysplastic lesions ($P<0.05$).
2. **HER2** expression was significant between the high-grade malignant tumors (Grade II and III) ($P<0.05$).
3. **PD-L1, ER-β,** and **PR** expression showed no significant differences between the groups and were not associated with survival outcomes.
4. **TNBC** cases showed increased expression of PD-L1 and CTLA-4 biomarkers, and this could suggest that these biomarkers may have potential for immune checkpoint-based therapeutic approaches.
5. **Tumor size** was not associated with biomarker expression, whereas NLR showed a relationship with PD-L1 expression, indicating a possible link between systemic inflammatory status and immune-related tumor behavior.

These findings provide additional insight into the immunological characteristics of canine mammary tumors and may support further research on immune checkpoint pathways and prognostic biomarkers.

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