THESIS

SIGNIFICANCE OF ERα, HER2, AND CAV1 EXPRESSION AND MOLECULAR SUBTYPE CLASSIFICATION TO CANINE MAMMARY GLAND TUMOR

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ABSTRACT

SIGNIFICANCE OF ERα, HER2, AND CAV1 EXPRESSION AND MOLECULAR SUBTYPE CLASSIFICATION TO CANINE MAMMARY GLAND TUMOR

Canine mammary gland tumor and human breast cancer share many similar features regarding their risk factors, histopathological features, and behavior. Despite the increasing evidence of molecular marker expression as a prognostic factor for human breast cancer, there are only little studies using this approach on canine mammary gland tumor. Our aim was to evaluate the significance of the expression of Estrogen Receptor-alpha, Human Epidermal Growth Factor-2, and Caveolin-1 to the behavior and the clinical outcome of canine mammary gland tumor by Immunohistochemistry. We also assessed the correlation between 5 subtype classification (Luminal A, Luminal B, HER2-overexpressing, Basal-like, and Normal-like) and tumor behavior and prognosis. Canine mammary gland tissues were stained for Estrogen Receptor-alpha, Human Epidermal Growth Factor-2, and Caveolin-1 and evaluated for the positivity, and classified into 5 subtypes according to the staining status. Although there was no statistical significance among the subtypes, the positivity of Nuclear Estrogen Receptor-alpha, Extranuclear Estrogen Receptor-alpha, Human Epidermal Growth Factor-2, and Caveolin-1 showed significant correlations ($p<0.05$) in the behavior and the prognosis of the tumor. This study indicates the prognostic value of immunohistochemistry staining status of Estrogen Receptor-alpha, Human Epidermal Growth Factor-2, and Caveolin-1 for canine mammary gland tumor. In addition, some trends were seen in 5 subtypes on the prognosis of the tumor, implying that although further analysis is needed, the potential application of 5 subtype classification to canine mammary gland tumor.

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INTRODUCTION

Canine mammary gland tumor (CMT) and human breast cancer (HBC) are both characterized as highly heterogeneous neoplasms regarding their clinical response and prognosis. Although histomorphological features are somewhat different between the two species, several studies using molecular markers have shown that CMT and HBC share significant similarities in the expression of these markers with regard to their histomorphological behavior and prognosis.[12, 29, 52, 54]

Despite attempts to investigate prognostic factors for CMT and HBC, there have been no definitive indicators to aide in determination of the clinical outcomes for both of these tumors. Recent approaches categorize HBC into 5 subtypes according to the expression of molecular markers have shown correlation with the behavior of the tumor.[13, 18, 20, 57, 63] These markers include Hormone Receptors (HR), such as Estrogen Receptor-alpha (ERα) and Progesterone Receptor (PR), Human Epidermal Growth Factor Receptor-2 (HER2), and Basal-like markers. This classification system categorizes the tumor according to positive or negative expression of these protein molecules into Luminal A subtype (HR+, HER2-, Basal markers+/-), Luminal B subtype (HR+, HER2+, Basal markers+/-), HER2-overexpressing subtype (HR-, HER2+, Basal markers+/-), Basal-like subtype (HR-, HER2-, Basal markers+), and Normal-like subtype (negative for all of the markers).[13, 18, 20, 57, 63] To our knowledge, there has been minimal research applying this classification to CMT.[7, 29, 54, 54]

Previous studies evaluating the molecular expression of ERα and PR in CMT have shown that the behavior of these two molecules is very similar.[22, 40] This can be explained by the expression of PR being strongly dependent on ERα.[27, 40] In addition, Immunohistochemistry (IHC) staining of PR is less consistent when compared to that of ERα.[26] In this study we used ERα as the

indicator for HR. ERα status has been shown to be very important in designing HBC target therapy. It is well known that ERα positive HBC has significantly better outcomes when treated with selective estrogen receptor modulators (SERM) and aromatase inhibitors.[5, 19, 23, 62] The expression of ERα in these tumors is also related to lower grade and higher survival rates.[25, 45] Hence, a major thrust of research, on both HBC and CMT, is to understand the relationship between ERα expression and tumor behavior. ERα localizes in the nucleus, plasma membrane, and cytoplasmic component.[41] Nuclear ERα (ERαN) regulates gene transcription by two pathways. In the direct/classical pathway, ERαN binds to the Estrogen Responsive element in the promoter region, and in the indirect/non-classical pathway, it binds to non- Estrogen Responsive element region and regulates transcription indirectly. Extranuclear ERα (ERαC) resides in plasma membrane or cytoplasm. Ligand binding activates the membrane ERα, which translocates into the nucleus or initiates signaling cascade to activate ERαN. Cytoplasmic ERα is found in mitochondria and is thought to maintain the integrity of the mitochondrial membrane and prevent intrinsic cell death.[42, 64]

HER2 is a member of Human Epidermal Growth Factor Receptor family, which also includes HER1 (EGFR), HER3, and HER4. In physiological conditions, ligand binding initiates HER family dimerization. Members of the HER family form either homo- or heterodimers and activate signaling pathways for cell proliferation and survival. Overexpression of HER2 leads to excessive HER2 dimers without the presence of ligand and plays a role in the growth and resistance to treatment for many types of cancer.[4, 35] HER2 expression has been used to predict prognosis and treatment sensitivity to certain cancers. In human medicine, trastuzumab, a HER2 antagonist, has been shown to improve the prognosis significantly as a chemotherapeutic and adjuvant treatment agent for HER2 over-expressing metastatic HBC.[20, 30, 39, 61] Thus, IHC analysis

of HER2 expression is used extensively in clinical and laboratory studies on HBC, and in a more limited basis (primarily laboratory studies) on CMT.[21, 29, 30, 36, 39, 54, 61]

The term “Basal-like subtype” is implied for tumor cells expressing molecular markers characteristic of basal or myoepithelial cells. Thus, the Basal-like subtype is thought to be the more undifferentiated type of cancer, and a variety of molecular markers such as P-cadherin[9, 29, 47], p63[29, 47], fatty-acid-binding protein 7[1, 59], EGFR[3, 36], BRCA-1[6, 28], nestin[16, 37, 43], osteonectin[49], vimentin[11], laminin[38, 44], c-KIT[47], and cytokeratin markers [29, 26,47, 54] are used for classification of this particular subtype. However, despite extensive research, the definitive molecular marker for the Basal-like subtype has not been agreed upon.

Caveolins are plasma membrane proteins that control cell signaling by regulating membrane binding and intercellular transport of essential molecules, such as albumin, cholesterol, endothelial nitric oxide synthase, growth hormone, and insulin.[15] Caveolin-1 (CAV1), a member of Caveolin family is thought to have a significant role in tumorigenesis and is highly expressed in myoepithelial cells, endothelial cells, and adipocytes.[15, 55] Many HBC studies have found associations between the expression of CAV1 and Basal-like subtypes[24, 25, 26]

In this study, we evaluated the significance of the IHC expression of three molecular markers, ERα, HER2, and CAV1 to the behavior and prognosis of CMT by two perspectives; positivity of three markers, and a new subtype classification, similar to that used for HBC.

MATERIALS AND METHODS

Samples

Tissues from 73 canine mammary gland surgical biopsies that were submitted to Colorado State University Veterinary Diagnostic Laboratory from 05/31/2001 to 07/30/2003 were used in this study. All tissues were diagnosed as CMT. Tissues were fixed in 10% buffer formalin solution, processed and embedded into paraffin using standard procedures, sectioned into 5µm sections using a Leica RM2255 rotary microtome (Leica Microsystems Inc., Buffalo Grove, IL) and mounted on glass slides.

Immunohistochemistry

5µm consecutive sections from formalin fixed paraffin embedded blocks of CMT tissues were tested for the presence of ERα, HER2, and CAV1 by IHC. The following primary antibodies were used; Monoclonal Mouse Anti-human ERα, Clone-1D5 (DAKO, Carpinteria, CA), Polyclonal Rabbit Anti-human HER2, C-18 (Santa Cruz Biotechnology, Dallas, TX), and Polyclonal Rabbit Anti-human CAV1, N-20 (Santa Cruz Biotechnology, Dallas, TX). Canine uterine tissue, human mammary carcinoma, and capillary endothelial cells were used as positive controls for ERα, HER2, and CAV1 respectively. Primary antibody incubation was omitted to provide a negative control for each sample.

Immunohistochemistry for ERα

Sections were deparaffinized and rehydrated in xylene and in descending concentrations of EtOH. ERα antigen was retrieved with antigen retrieval solution ph9.0 (DAKO, Carpinteria, CA) in a pressure cooker (Biocare Medical, Concord, CA) for 1 min. at 125ºc. Slides were cooled in distilled (DI) water for 1min, placed in Sequenza system (Thermo Fisher Scientific, Waltham, MA) and Background sniper (Biocare Medical, Concord, CA) and hydrogen peroxide were used

to block nonspecific staining. Following 1:50 diluted Monoclonal Mouse Anti-Human ERα Clone-1D5 (DAKO, Carpinteria, CA) overnight at 4ºc, slides were incubated with EnVision+ Dual Link System-HRP (DAKO, Carpinteria, CA). 3', 3'-diaminobenzidine (DAB) (Vector Laboratories, Burlingame, CA) was used to visualize staining and hematoxylin was used for the counter-stain. Finally, slides were dehydrated with ascending concentrations of EtOH and xylene, mounted, and coverslipped. Slides were washed with buffered saline between procedures. All procedures were performed at room temperature unless indicated.

Immunohistochemistry for HER2 and CAV1

Sections were deparaffinized and rehydrated in xylene and in descending concentrations of EtOH. HER2 and CAV1 antigens were retrieved with Citra solution (Bio Genex, San Ramon, CA) by microwaving for 2min 30sec at 700W and then for 10min at 200W. After antigen retrieval, slides were cooled in DI water and placed in Hi Pro slide incubator (Thermo Fisher Scientific, Waltham, MA). Slides were incubated with Vector Elite diluted goat serum (Vector Laboratories, Burlingame, CA) and hydrogen peroxide to block background staining, then labeled with 1:500 diluted Polyclonal Rabbit Anti-human HER-2, C-18 (Santa Cruz Biotechnology, Dallas, TX) or 1:100 diluted Polyclonal Rabbit Anti-human CAV1, N-20 (Santa Cruz Biotechnology, Dallas, TX) for 15min. Following 30 min incubation with Vector Elite biotinylated anti-mouse/rabbit secondary antibody (Vector Laboratories, Burlingame, CA), slides were incubated with Vector Elite avidin biotinylated horseradish peroxidase complex (Vector Laboratories, Burlingame, CA) for 30min. Slides were visualized with 3-amino-9-ethyl carbazole (AEC) (Biomeda, Foster City, CA) and counter-stained with hematoxylin. Finally, slides were dehydrated with ascending concentrations of EtOH and xylene, mounted, and coverslipped. Slides were washed with buffered saline between procedures. All procedures were performed at 37ºC, unless indicated.

Evaluation for ERα

IHC slides were examined for ERα expression for both nuclear and extranuclear component. Tissues were considered positive for ERαN and/or ERαC when ≥ 5% of tumor cells were stained for nuclei and membrane/cytoplasm, respectively. This scoring system is the most commonly used method for IHC studies on CMT.[45, 53, 55] The status for ERαN staining was used as described in the HBC 5 subtype classification.[7, 13, 20, 22, 45, 48, 54, 55, 57, 63]

Evaluation for HER2

We applied the FDA-approved scoring system for HBC, which is utilized in the HER2 expression Hercep Test (DAKO, Carpinteria, CA) to evaluate the overexpression of HER2. The scoring system interprets IHC staining as HER2 positive (3+) for intense membrane staining of > 30% of tumor cells, equivocal (2+) for complete membrane staining that is either non-uniform or weak in intensity but with obvious circumferential distribution in ≥ 10% of tumor cells, and negative (0 or 1+) for no staining or weak/incomplete membrane staining regardless of the distribution of stained tumor cells. In this study, we classified 3+ stained samples as positive and 2+, 1+, and 0 samples as negative.

Evaluation for CAV1

Slides were evaluated for CAV1 expression using the semi-quantitative scoring system described previously.[55] Samples were evaluated for staining intensity (0; none, 1; weakly positive, 2; moderately positive, and 3; strongly positive) and the distribution of positively stained tumor cells (0; < 1%, 1; 1-9%, 2; 10-24%, 3; 25-49%, 4; 50-100%).

The samples were considered positive if the sum of the scores for the staining intensity and the distribution of stained tumor cells were ≥ 4. This scoring system was described as being more significantly related to the survival period than methods used in other studies.[51]

5 Subtype Classifications

All samples were classified into 5 subtypes according to the expression of three molecular markers; ERα, HER2, and CAV1 (Table 1).

Table 1: 5 Subtype Classification

	ERαN	HER2	CAV1
Luminal A	+	-	±
Luminal B	+	+	±
HER2-overexpressing	-	+	±
Basal-like	-	-	+
Normal-like	-	-	-

ERαN=Nuclear Estrogen Receptor-alpha, HER2=Human Epidermal Growth Factor Receptor-2, CAV1=Caveolin-1, ±=regardless of the positivity.

History

The primary report for each case was reviewed for sex, age at the time of surgery, and alteration status. In addition, a follow-up survey to assess patient prognosis was obtained from the referring veterinarian, which included recurrence of the tumor and the time of death.

Histopathologic Evaluation

Slides were stained with Hematoxylin and Eosin and evaluated for growth pattern (ductular, papillary, solid), invasion pattern (expansile, local, regional, nodal, vascular), percent necrosis, mitotic index (number of cells with mitotic figure per 10 high power fields from the neoplastic area with mitotic activity), degree of schirrhous reaction, degree of anaplasia, and degree of

inflammation (0; none, 1: mild, 2: moderate, 3: marked).

Morphologic Classification

The morphologic diagnosis of the CMT was derived from the classification system developed by Benjamin SA et al.[8] The benign classification includes, adenoma simple (AdS), adenoma complex (AdC), benign mixed (BM), lobular hyperplasia (LH), ductular hyperplasia (DH), and ductular papilloma (DP). The malignant classification includes, adenocarcinoma simple (AS), adenocarcinoma complex (AC), ductular carcinoma (DC), and ductular papillary carcinoma (DPC). Tumors that were too poorly differentiated to be diagnosed morphologically were classified as solid carcinoma (SC). Simple adenoma/adenocarcinoma has either epithelial or myoepithelial component, and complex adenoma/adenocarcinoma has both epithelial and myoepithelial component. All samples were also classified according to their morphologic origin, either lobular origin or ductular origin. Lobular origin includes, AdS, AdC, BM, LH, AS, and AC. Ductular origin includes, DH, DP, DC, and DPC. SC was excluded from either origin.[8] All microscopic evaluations were done by two veterinary pathologists.

Statistical Analysis

In this study, two different perspectives, (1) positivity of each molecular marker and (2) a 5 subtype classification scheme, were used to analyze the correlation with the behavior and the prognosis of the tumors. All statistical analyses were performed using GraphPad Prism version 4.00 (GraphPad Software, San Diego CA). To analyze the correlation with the positivity of each marker, Mann-Whitney test and Fisher's exact test were used, and the correlation with the 5 subtypes were analyzed by ANOVA (Kruskal-Wallis test) with Dunnett's post test. The Kaplan-Meier survival curve with Longrank test was used to analyze the disease free interval and the overall survival. The level of significance was fixed at $P < 0.05$.

RESULTS

Immunohistochemistry

73 cases were examined for the IHC staining of three markers, ERα, HER2, and CAV1 (Fig. 1). 35 cases (48%) were Nuclear ERαpositive (ERαN+) and 38 cases (52%) were Nuclear ERα negative (ERαN-). 59 cases (81%) were Extranuclear ERα positive (ERαC+) and 14 cases (19%) were Extranuclear ERα negative (ERαC-). 28 cases (38%) were HER2 positive (HER2+) and 45 cases (52%) were HER2 negative (HER2-). 60 cases (82%) were CAV1 positive (CAV1+) and 13 cases (18%) were CAV1 negative (CAV1-) (Table 2). Extranuclear ERα staining was either completely positive or completely negative.

We classified 73 cases into 5 molecular subtypes according to the expression of three markers; this subtype classification scheme is a modification of the published HBC scheme [13, 18, 20, 57, 63] and used ERα markers for HR in CMT samples instead of ERα and PR used for HRs in evaluating HBC. 18 cases (25%) were classified as Luminal A subtype, 17 cases (23%) were classified as Luminal B subtype, 11 cases (15%) were classified as HER2-overexpressing subtype, 24 cases (33%) were classified as Basal-like subtype, and 3 cases (4%) were classified as Normal-like subtype (Table 3).

History

All 73 cases were females, 21 (29%) spayed and 52 (71%) intact. There were more intact than spayed females regardless of molecular marker staining or tumor subtype. The age of onset was obtained from 67 cases. The mean age was 8.6 years old and there was no significant age difference between marker positivity and among subtypes (Table 3).

Follow-up surveys were obtained from 69 cases with the longest follow-up period of 2942 days after diagnosis. Out of these cases, 27 cases (39%) had recurrence lesion and 31 (42%) cases

were deceased at the time of the report. ERαC+ tumors (44%) had significantly higher (p=0.011) recurrence rate than ERαC- tumors (8%), and HER2+ tumors (29%) had significantly lower (p=0.019) recurrence rate than HER2- tumors (43%). There were no significant differences in percentage of recurrence among other markers or among the subtypes. The median (50%) disease free interval and the median (50%) overall survival for all 69 cases were 650 days and 1333 days, respectively. ERαN- tumors had the shortest (584 days) and CAV1- tumors had the longest (1709 days) median (50%) disease free interval. ERαN- tumors had the shortest (788 days) and HER2+ tumors had the longest (1509 days) median (50%) overall survival (Table 2). In terms of subtype, HER2 subtype had the lowest percentage of recurrence (18%), Basal-like subtype had the shortest (584 days) and Normal-like subtype had the longest (1709 days) median (50%) disease free interval. Normal-like subtype had the shortest (224 days) and HER2 subtype had the longest (2942 days) median (50%) overall survival (Table 3). There were no significant differences between the positivity of markers among the subtypes. (Fig. 2)

Table 2: History and follow-up information-marker status

	ERαN+	ERαN-	ERαC+	ERαC-	HER2+	HER2-	CAV1+	CAV1-
Number of Cases*	35 (48%)	38 (52%)	59 (81%)	14 (19%)	28 (38%)	45 (62%)	60 (82%)	13 (18%)
Mean Age (year)	8.6	8.6	8.5	9.1	8.4	8.7	8.5	9.2
Spayed†	11 (32%)	10 (26%)	18 (31%)	3 (21%)	11 (39%)	10 (22%)	16 (27%)	5 (38%)
Intact†	24 (68%)	28 (74%)	41 (69%)	11 (79%)	17 (51%)	35 (78%)	44 (73%)	8 (62%)
Follow-up cases¶	34 (49%)	35 (51%)	57 (82%)	12 (18%)	27 (39%)	42 (61%)	56 (81%)	13 (19%)
Recurrent cases‡	14 (41%)	12 (34%)	25 (44%)	1 (8%)	8 (29%)	18 (43%)	23 (50%)	3 (23%)
DF (day)	650	584	650	**	**	650	650	1709
OS (day)	1333	788	1333	**	1509	1284	1333	1371

* Parentheses indicate % out of total 73 cases.

†Parentheses indicate % out of corresponding number of cases.

¶Parentheses indicate % out of total 69 follow-up cases.

‡Parentheses indicate % of recurrent cases out of corresponding follow-up cases.

** Undefined data due to small number of recurrent or deceased cases.

DF=Median Disease free interval, OS=Median Overall survival, ERαN+=Nuclear Estrogen Receptor-alpha positive, ERαN-=Nuclear Estrogen Receptor-alpha negative, ERαC+=Extranuclear Estrogen Receptor-alpha positive, ERαC-=Extranuclear Estrogen Receptor-alpha negative, HER2+=Human Epidermal Growth Factor Receptor-2 positive, HER2-=Human Epidermal Growth Factor Receptor-2 negative, CAV1+=Caveolin-1 positive, CAV1-=Caveolin-1 negative.

Table 3: History and follow-up information-subtype

	Luminal A	Luminal B	HER2	Basal	Normal	Total
Number of Cases*	18 (25%)	17 (23%)	11 (15%)	24 (33%)	3 (4%)	73
Mean Age (year)	8.9	8.4	8.6	8.6	8.7	8.6
Spayed†	4 (22%)	7 (41%)	4 (36%)	5 (21%)	1 (33%)	21 (29%)
Intact†	14 (78%)	10 (59%)	7 (64%)	19 (79%)	2 (67%)	52 (71%)
Follow-up cases¶	18 (26%)	16 (23%)	11 (16%)	20 (29%)	2 (3%)	69
Recurrent cases‡	8 (44%)	6 (38%)	2 (18%)	10 (50%)	1 (50%)	27 (39%)
DF (day)	650	**	**	584	1709	650
OS (day)	1284	1485	2942	733	224	1333

* Parentheses indicate % out of total 73 cases.

†Parentheses indicate % out of corresponding number of cases.

¶Parentheses indicate % out of total 69 follow-up cases.

‡Parentheses indicate % of recurrent cases out of corresponding follow-up cases.

** Undefined data due to small number of recurrent or deceased cases.

DF=Median Disease free interval, OS=Median Overall survival, Luminal A=Luminal A subtype, Luminal B=Luminal B subtype, HER2=HER2-overexpressing subtype, Basal=Basal-like subtype, Normal=Normal-like subtype.

Histopathologic Evaluation

Analysis of growth pattern showed the highest percentage were of the ductular pattern regardless of the positivity of the markers. For invasion, the expansile pattern was the most common pattern except for the ERαC- tumors, which did not have a distinctive dominant pattern (Table 3-1). Luminal A subtype (50%), Luminal B subtype (65%), HER2 subtype (46%), and Basal-like subtype (66%) had the ductular pattern as the highest percentage. Luminal A subtype (41%), Luminal B subtype (47%), and Basal-like subtype (50%) had the largest distribution of the expansile pattern, and HER2 subtype had expansile (40%) and vascular (40%) as the

predominant invasion patterns (Table 5). There were no significant differences in the growth and invasion pattern between the positivity of the markers and among the subtypes. The percent necrosis of ERαC+ tumors (7.7%) was significantly lower ($p=0.048$) than that of ERαC- tumors (20.6%). Degree of anaplasia was significantly lower ($p=0.041$) in CAV1+ tumors than CAV1- tumors. There were no significant differences between the positivity of markers and among the 5 subtypes based on other histopathologic criteria.

Table 4: Histopathologic evaluation-marker status

		ERαN+	ERαN-	ERαC+	ERαC-	HER2+	HER2-	CAV1+	CAV1-
Number of Cases*		35 (48%)	38 (52%)	59 (81%)	14 (19%)	28 (38%)	45 (62%)	60 (82%)	13 (18%)
Growth†	Solid	8 (23%)	8 (21%)	10 (17%)	6 (43%)	8 (29%)	8 (18%)	12 (21%)	4 (31%)
	Papillary	7 (20%)	8 (21%)	13 (22%)	2 (14%)	4 (14%)	11 (24%)	10 (17%)	3 (23%)
	Ductular	20 (57%)	22 (58%)	36 (61%)	6 (43%)	16 (57%)	26 (58%)	36 (62%)	6 (46%)
Invasion†	Expansile	15 (43%)	16 (42%)	27 (49%)	2 (17%)	12 (44%)	19 (45%)	26 (46%)	5 (38%)
	Local	8 (23%)	7 (18%)	12 (22%)	3 (25%)	5 (19%)	10 (24%)	13 (23%)	2 (15%)
	Regional	5 (14%)	5 (13%)	7 (13%)	3 (25%)	2 (7%)	8 (19%)	7 (12%)	3 (23%)
	Vascular	6 (17%)	7 (18%)	9 (16%)	4 (33%)	8 (30%)	5 (12%)	10 (18%)	3 (23%)
% Necrosis#		16.4	15.6	14.2	22.9	18.2	14.3	15.2	18.9
Mitotic Index#		9.0	11.2	7.7	20.6	12.3	8.9	9.3	14.3
Schirrhous Reaction#		1.5	1.6	1.6	1.4	1.6	1.4	1.5	1.8
Anaplasia#		1.5	1.4	1.6	1.4	1.4	1.6	1.5	1.8
Inflammation#		1.4	1.6	1.6	1.2	1.5	1.1	1.5	1.7

* Parentheses indicate % out of total 73 cases.

†Parenthesis indicate % out of corresponding number of cases.

#Average.

ERαN+=Nuclear Estrogen Receptor-alpha positive, ERαN-=Nuclear Estrogen Receptor-alpha negative, ERαC+=Extranuclear Estrogen Receptor-alpha positive, ERαC-=Extranuclear Estrogen Receptor-alpha negative, HER2+=Human Epidermal Growth Factor Receptor-2 positive, HER2-=Human Epidermal Growth Factor Receptor-2 negative, CAV1+=Caveolin-1 positive, CAV1-=Caveolin-1 negative.

Table 5: Histopathologic evaluation-subtypes

		Luminal A	Luminal B	HER2	Basal	Normal	Total
Number of Cases		18 (25%)	17 (23%)	11 (15%)	24 (33%)	3 (4%)	73
Growth†	Solid	3 (17%)	5 (29%)	3 (27%)	4 (17%)	1 (33%)	16 (22%)
	Papillary	6 (33%)	1 (6%)	3 (27%)	4 (17%)	1 (33%)	15 (21%)
	Ductular	9 (50%)	11 (65%)	5 (46%)	16 (66%)	1 (33%)	42 (57%[
Invasion†	Expansile	7 (41%)	8 (47%)	4 (40%)	11 (50%)	1 (33%)	31 (45%)
	Local	4 (24%)	4 (24%)	1 (10%)	6 (27%)	0 (0%)	15 (22%)
	Regional	4 (24%)	1 (6%)	1 (10%)	3 (14%)	1 (33%)	10 (14%)
	Vascular	2 (12%)	4 (24%)	4 (40%)	2 (9%)	1 (33%)	13 (19%)
% Necrosis#		14.7	18.2	18.2	13.8	16.7	15.8
Mitotic Index#		8.7	9.3	16.8	5.3	38.7	10.2
Schirrhous Reaction#		1.3	1.6	1.7	1.5	1.3	1.5
Anaplasia#		1.4	1.6	1.7	1.3	1.7	1.5
Inflammation#		1.6	1.3	1.6	1.6	1.7	1.5

* Parentheses indicate % out of total 73 cases.

†Parenthesis indicate % out of corresponding number of cases.

#Average.

Luminal B=Luminal B subtype, HER2=HER2-overexpressing subtype, Basal=Basal-like subtype, Normal=Normal-like subtype.

Morphologic Classification

Adenocarcinoma Simple was the most common morphological type among the 73 samples. There were significant differences in the trend of morphological pattern for positivity of ERαN ($p<0.001$) and ERαC ($p<0.0001$) (Table 6, Fig. 3). ERαN+ had higher distribution of AdC, DPC, and SC, where ERαN- had higher distribution of LH, AS, and DC. ERαC+ had higher distribution of AdS, AdC, DH, DC, and DPC, and ERαC- had higher distribution of LH, DP, AS, and SC. There were no significant differences among subtypes regarding the morphological pattern. (Table 7, Fig. 4) When dividing the morphological pattern into lobular origin and ductular origin, the lobular origin had much higher percentage overall, regardless of the markers' positivity or subtypes (Table 6, Table 7, Fig. 3, Fig. 4). The distribution of benign tumor was slightly lower than that of malignant tumor without regards to the positivity of the markers or subtypes, though no statistical significant were observed (Table 6, Table 7, Fig. 3, Fig. 4).

Table 6: Distribution of morphological type indicated in % out of corresponding marker status

	ERαN+	ERαN-	ERαC+	ERαC-	HER2+	HER2-	CAV1+	CAV1-
Number of Cases	35	38	59	14	28	45	60	13
AdS	6%	5%	7%	0%	4%	9%	7%	0%
AdC	17%	8%	15%	0%	14%	7%	13%	8%
BM	17%	18%	17%	21%	25%	11%	20%	8%
LH	0%	8%	3%	7%	4%	13%	2%	15%
DH	3%	3%	3%	0%	0%	4%	3%	0%
DP	0%	3%	0%	7%	0%	4%	0%	8%
AS	20%	26%	22%	29%	25%	2%	27%	8%
AC	11%	8%	10%	7%	7%	22%	10%	8%
DC	3%	11%	8%	0%	4%	11%	5%	15%
DPC	6%	3%	5%	0%	0%	9%	3%	8%
SC	17%	8%	8%	29%	18%	7%	10%	23%
Lobular	86%	80%	79%	90%	96%	76%	87%	60%
Ductular	14%	20%	21%	10%	4%	24%	13%	40%
Benign	41%	45%	46%	36%	46%	42%	45%	38%
Malignant	59%	55%	54%	64%	54%	58%	55%	62%

ERαN+= Nuclear Estrogen Receptor-alpha positive, ERαN-= Nuclear Estrogen Receptor-alpha negative, ERαC+= Extranuclear Estrogen Receptor-alpha positive, ERαC-= Extranuclear Estrogen Receptor-alpha negative, HER2+= Human Epidermal Growth Factor Receptor-2 positive, HER2-= Human Epidermal Growth Factor Receptor-2 negative, CAV1+= Caveolin-1 positive, CAV1-= Caveolin-1 negative.
AdS= adenoma simple, AdC= adenoma complex, BM= benign mixed, LH= lobular hyperplasia, DH= ductular hyperplasia, DP= ductular papilloma, AS= adenocarcinoma simple, AC= adenocarcinoma complex, DC= ductular carcinoma, DPC= ductular papillary carcinoma, SC= solid carcinoma.

Table 7: Distribution of morphological type indicated in % out of corresponding subtype

	Luminal A	Luminal B	HER2	Basal	Normal	Total
Number of Cases	18	17	11	24	3	73
AdS	11%	0%	8%	4%	0%	4%
AdC	11%	24%	0%	13%	0%	9%
BM	11%	24%	25%	17%	0%	13%
LH	0%	0%	8%	4%	33%	3%
DH	6%	0%	0%	4%	0%	2%
DP	0%	0%	8%	0%	0%	1%
AS	22%	18%	33%	26%	0%	17%
AC	11%	12%	0%	13%	0%	7%
DC	6%	0%	8%	13%	0%	5%
DPC	11%	0%	0%	0%	33%	3%
SC	11%	24%	8%	4%	33%	9%
Lobular	75%	100%	82%	82%	50%	83%
Ductular	25%	0%	12%	18%	50%	17%
Benign	39%	47%	45%	42%	33%	42%
Malignant	61%	53%	55%	58%	67%	58%

Luminal A= Luminal A subtype, Luminal B= Luminal B subtype, HER2= HER2-overexpressing subtype, Basal= Basal-like subtype, Normal= Normal-like subtype.
AdS= adenoma simple, AdC= adenoma complex, BM= benign mixed, LH= lobular hyperplasia, DH= ductular hyperplasia, DP= ductular papilloma, AS= adenocarcinoma simple, AC= adenocarcinoma complex, DC= ductular carcinoma, DPC= ductular papillary carcinoma, SC= solid carcinoma.

DISCUSSION

The distribution for positively stained tumors of ERαN (48%), HER2 (38%), and CAV1 (82%) was consistent with previous IHC studies on CMT.[2, 7, 29] Our results showed a high proportion of ERαC+ staining (82%). However, in our knowledge, there is no canine study reporting IHC staining on ERαC. When we classified the tumors into 5 subtypes according to IHC staining, Basal-like subtype (24 cases, 33%) was the most common subtype, followed by Luminal A subtype (18 cases, 25%), Luminal B subtype (17 cases, 23%), HER2-overexpressing subtype (11 cases, 15%), and Normal-like subtype (3 cases, 4%). Among the few studies on CMT that use a subtype classification with IHC, Gama et al.[29] used only ERα as the hormone receptor. The distribution of CMT for each subtype in their study was 44.8% Luminal A subtype, 13.5% Luminal B subtype, 8.3% HER2 subtype, 29.2% Basal-like subtype, and 4.2% Negative/null (equivalent to Normal-like, in this study).[29] Compared to the Gama study, our results showed a lower distribution of Luminal A subtype and higher distribution of Luminal B subtype. This could be explained by the higher positivity of HER2 (38%) in our study compared to Gama's study, which may be due to using primary antibodies from different manufacturers, though both studies used the same scoring system. Most of other previous studies on CMT applying the same classification method have a higher percentage of Luminal subtypes.[7, 29, 53, 54] The factors contributing to the discrepancy in subtype distribution may be the different molecules used for the basal cell marker. "Basal-like" was named originally for HBC which indicates its transcriptome is similar to that of basal/myoepithelial cells.[24] However, there are numbers of candidates for this particular subtype because of the abundance of markers for these cell type. For example in CMT studies, Gama et al. used P-cadherin, Sassi et al. used CK 5/6, and Beha et al. used CK 5/6, CK14, or p63.[7, 29, 54] The lack of consistency in markers used for Basal-like subtype has been discussed

previously,[58] but there is no set definition despite the pursuit to identify the molecule that represents this subtype. We used CAV1 as the marker for Basal-like subtype since it is strongly expressed in myoepithelial cells and epithelial cells of normal human breast tissue and canine mammary tissue.[2, 51] Many human and canine research found that the correlation between CAV1 expression and prognosis varies among tumor types, that is to say, CAV1 overexpression indicates either better or worse prognosis depending on the type of cancer.[2, 51, 55] CAV1 is one of the components of caveolae, which is the membrane invaginations for various cell types and CAV1 acts as an anchor for many signaling molecules and regulates important cellular signaling cascades that relate to cell proliferation and survival.[15] Our data suggested that loss of CAV1 expression is significantly associated with higher degree of anaplasia. Loss of CAV1 function may lead to disruption of organized signaling pathway that are important in cell growth and metabolism, which results in loss of cellular differentiation and organization. Pireira et al. evaluated the changes in the IHC staining of CAV1 in cell types in CMT tissue and found that CAV1 is expressed in either luminal epithelial cells or myoepithelial cells depending on the malignancy of the tumor.[51] We did not distinguish the type of cell that were stained with CAV1 in this study. However, differentiating cell types with CAV1 status may uncover stronger association of CMT with CAV1 expression and/or subtypes. Another factor that plays into the lower population of Luminal subtypes in our result compared to previous studies[7, 29, 53, 54] may be the use of ERα as the sole marker for the HR. Many studies use ERα and PR expression when evaluating HR status on CMT. However, in human medicine, ERα is the only hormone receptor that is proven to have significant effect on clinical treatment and prognosis for HBC.[23] Moreover, the behavior of PR is strongly dependent on ER, and the IHC staining status of PR is less consistent.[26, 40] Hence, it is logical to use ERα as the indicator for the HR.

ERα status showed strong statistical significance to the distribution of morphological types. Toniti et al. stated that human, dog, and cat share the same major binding site for ERα to its endogenous ligands and SERM.[60] The strong association between ERα and morphology may suggest the possibility of determining ERα status by routine microscopic diagnosis for future prognostic factor or treatment decision on CMT. The result of the follow-up survey revealed that the shortest median disease free interval and median overall survival was for tumors staining ERαN-. The loss of hormone dependency corresponding to unfavorable outcome in CMT has been described previously, as tumors without ERαN expression have a shorter disease free interval[48] and overall survival.[45] The evaluation of positivity of the ERαC indicated that ERαC+ had significantly lower percent necrosis and significantly higher recurrence rate than ERαC-. There is evidence that membrane ERα interacts with transmembrane receptors, which trigger signaling cascade that contributes to phosphorylation of enzymes and ligand-independent activation of ERαN.[42, 46] In addition, cytoplasmic ERα prevents intrinsic cell death that is initiated by mitochondrial disruption.[42] Thus, it can be predicted that presence of ERαC contributes to the integrity of tumor cells. In other words, over expression of this marker might lead to higher susceptibility and loss of control of the cell cycle, perhaps leading to a higher recurrence rate. The importance ERαC function in HBC is attracting more attention in human research, but the results are controversial.[31, 42, 50]

Recent studies have shown that membrane ERα may set in caveolae, and therefore function of ERα might be related to CAV1 activity.[10, 14] Nevertheless, there is no research on CMT regarding the relationship between ERα and CAV1 nor the cross talk between ERαN and ERαC. Our result did not show correlation between the expression of these markers, but revealing the behavior and interaction between the markers would open a new understanding on CMT diagnosis

and treatment.

The recurrence rate for HER2+ tumor was significantly lower than HER2- tumor. In addition, HER2-overexpressing subtype had the lowest recurrence rate (18%) than any other subtypes (Luminal A; 44%, Luminal B; 38%, Basal-like; 50%, Normal-like; 50%). These results contradict HBC research, where HER2 overexpressing HBC has a poor prognosis, including higher recurrence rates.[56] However, IHC studies on CMT revealed HER2 expression was associated with a better prognosis. [28, 34] This disparity leads to the possibility of different roles for HER2 between HBC and CMT.

Although some trends were seen, current data was not statistically strong enough to support the correlation between the 5 subtypes and behavior or prognosis of CMT. However, there are still needs for further assessment of the expression of three markers in association with CMT. Finally, some contradicting results between HBC and CMT lead us to postulate careful interpretation of CMT research when used as a model for HBC, though these two species share many common features regarding this type of neoplasia.[29, 45, 55]

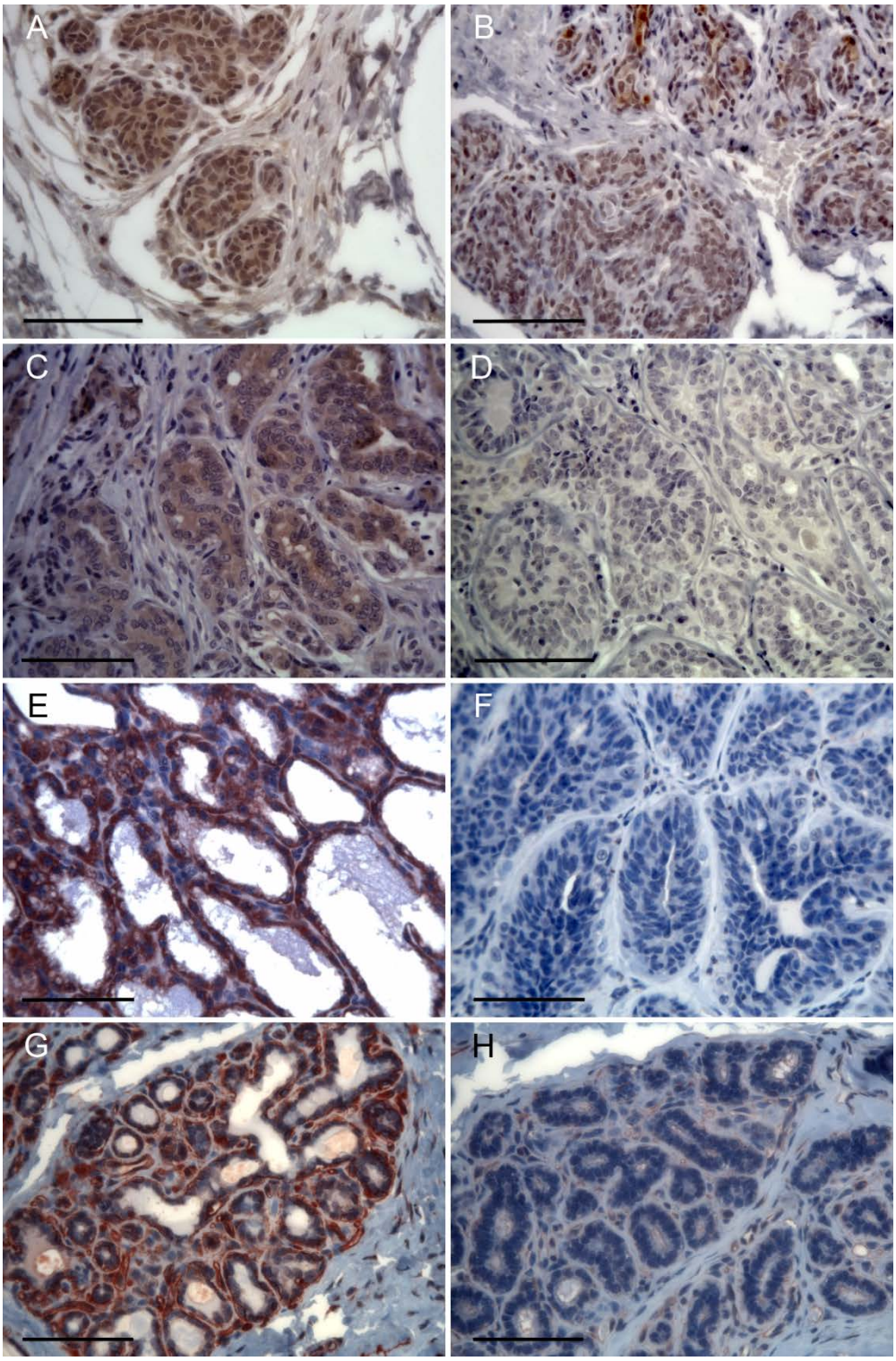

Fig. 1: Immunohistochemistry staining status of CMT.

Bar = 50μm. **(A)** ERαN+, ERαC+, **(B)** ERαN+, ERαC-, **(C)** ERαN-, ERαC+, **(D)** ERαN-, ERαC-, **(E)** HER2+, **(F)** HER2-, **(G)** CAV1+, **(H)** CAV1-.

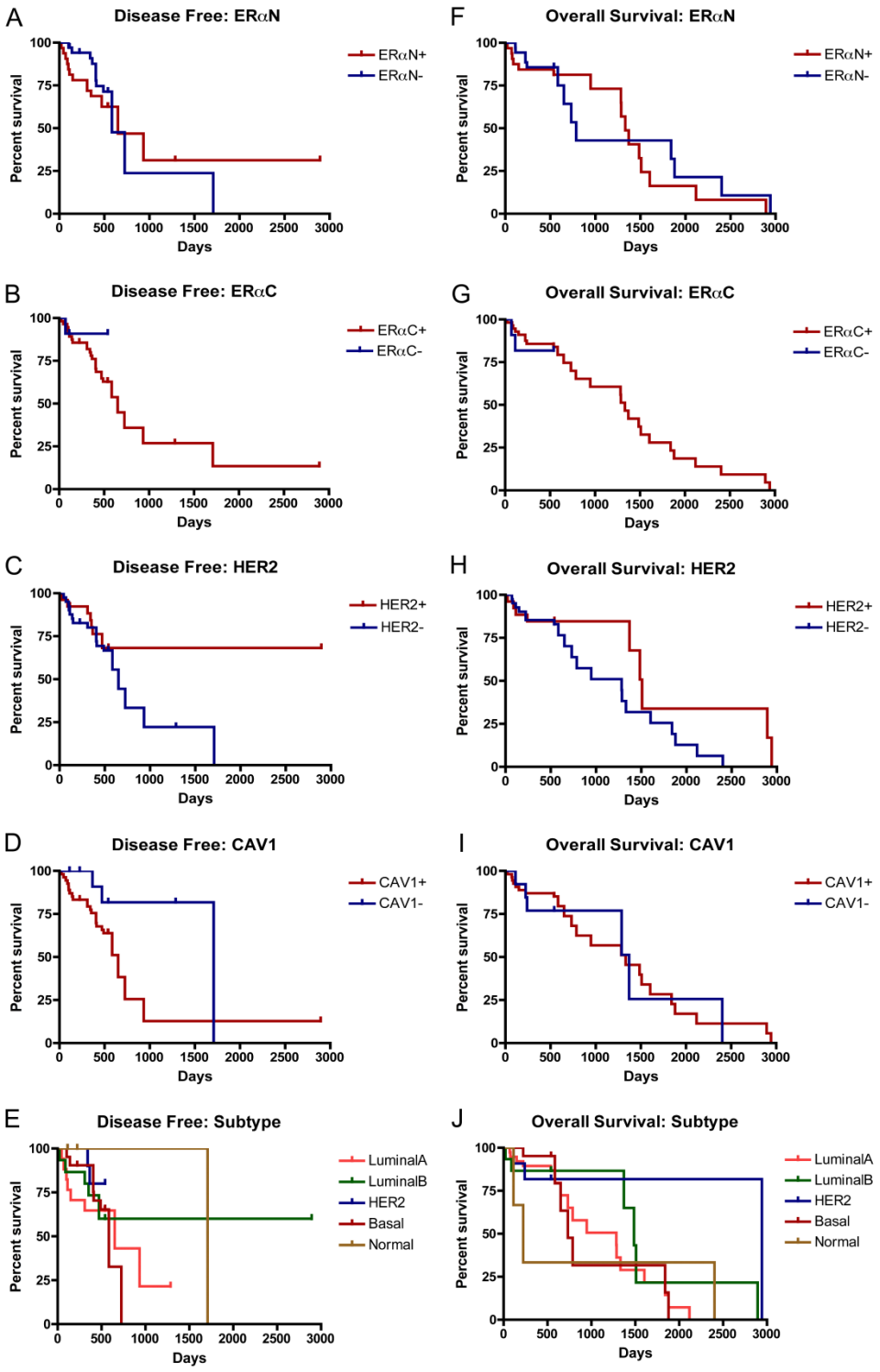

Fig. 2: Disease free interval and Overall survival.

(A)-(E) Disease free intervals. **(A)** ERαN, **(B)** ERα, **(C)** HER2, **(D)** CAV1, **(E)** 5 subtypes.

(F)-(J) Overall survivals. **(F)** ERαN, **(G)** ERαC, **(H)** HER2, **(I)** CAV1, **(J)** 5 subtypes

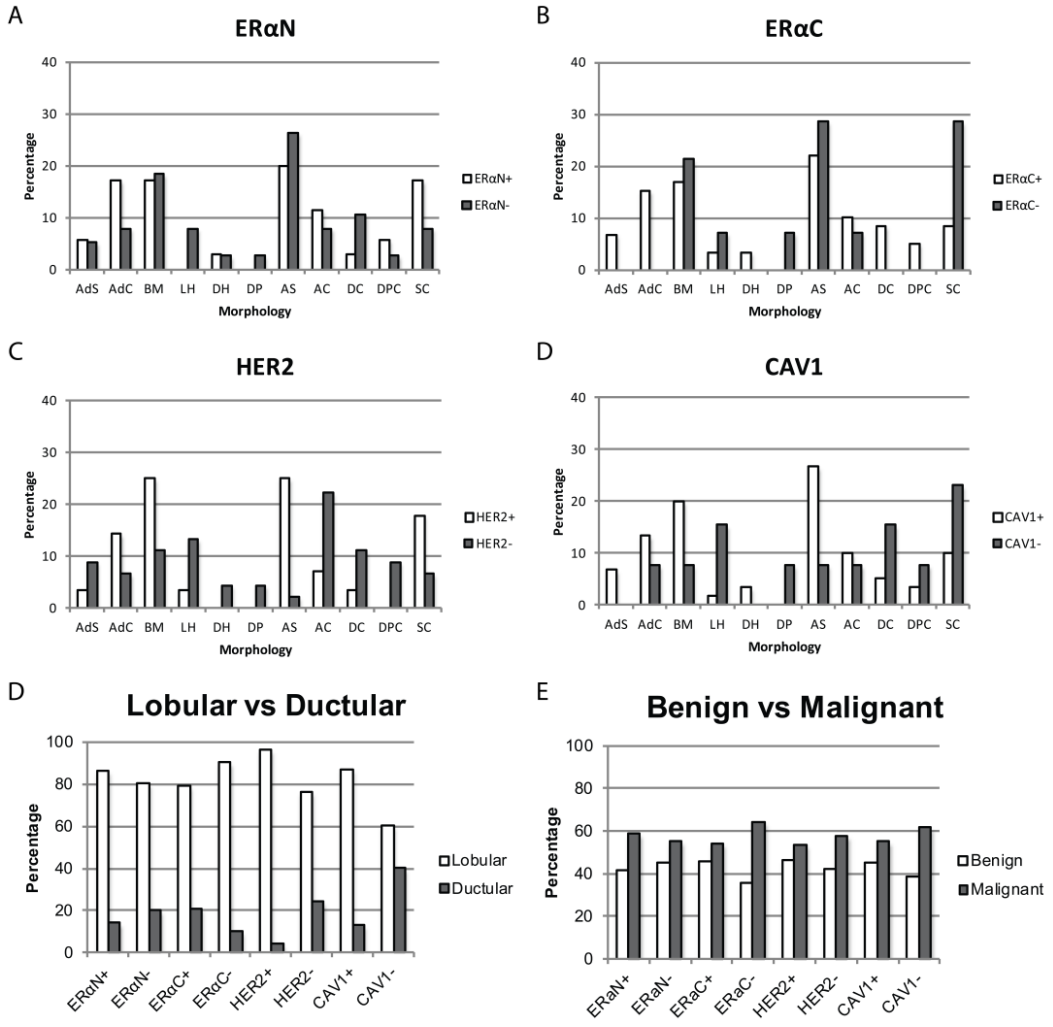

Fig. 3: Morphological evaluation according to molecular marker expression

(A)-(D) Distribution of morphological classification. **(A)** ERαN, **(B)** ERα, **(C)** HER2, **(D)** CAV1. **(G)** Distribution of morphological origin, **(H)** Distribution of malignancy.

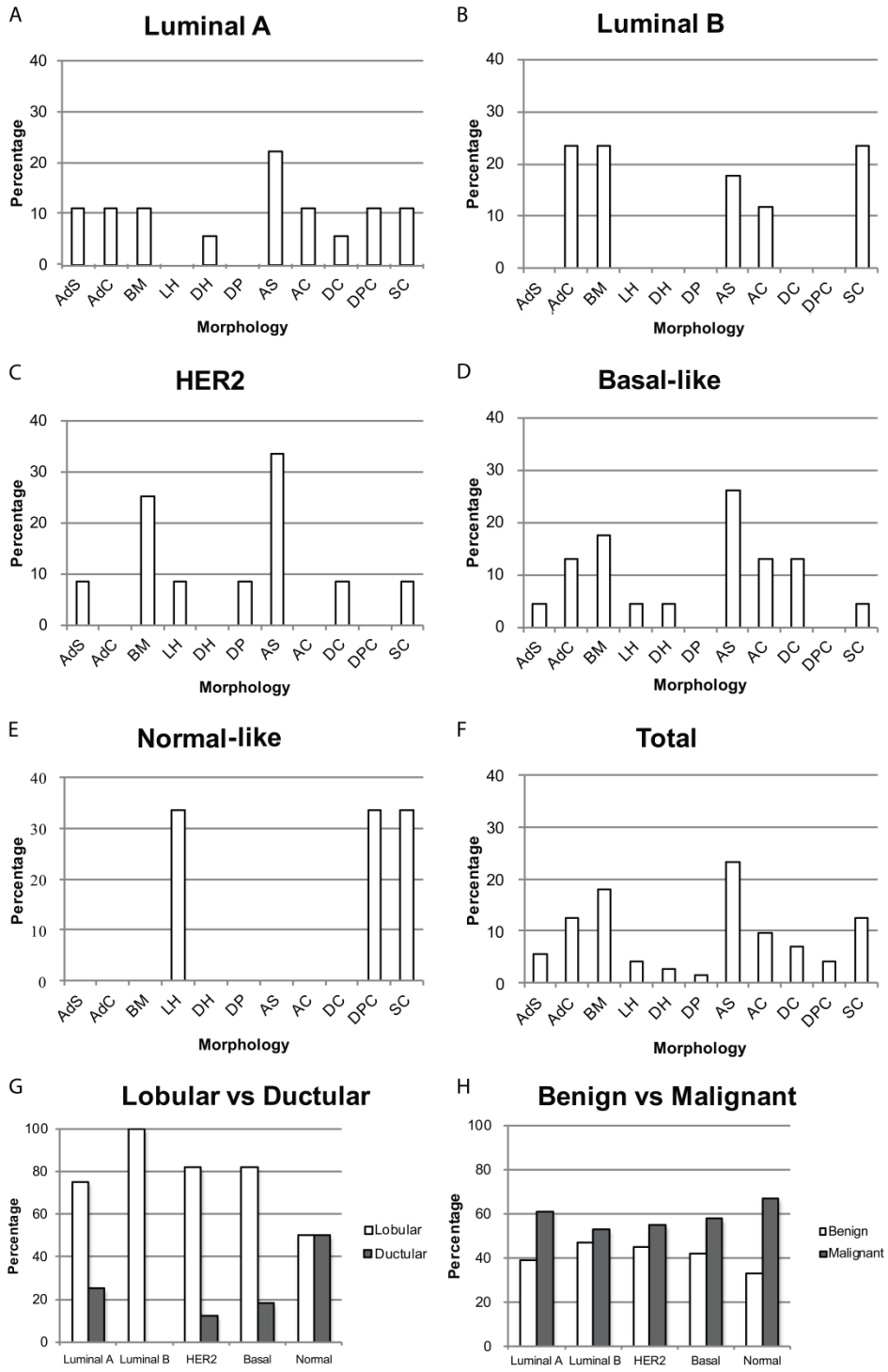

Fig. 4: Morphological evaluation according to 5 subtypes

(A)-(F) Distribution of morphological classification. **(A)** Luminal A subtype, **(B)** Luminal B subtype, **(C)** HER2-overexpressing subtype, **(D)** Basal-like subtype, **(E)** Normal-like subtype**, (F)** Total.
(G) Distribution of morphological origin, **(H)** Distribution of malignancy.

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