Calretinin expression as a risk biomarker for metastatic canine mammary carcinoma

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ABSTRACT

Malignant breast tumors are the most common tumors in humans and are associated with a poor prognosis. An accurate animal model of human mammary gland tumorigenesis is needed to test novel diagnosis and treatment strategies. Dogs represent a promising model since they develop such tumors spontaneously. In the present study, three immunomarkers, including calretinin, c-Kit (CD117) and placental alkaline phosphatase (Plap), were used and compared with each other, in relation to estrogen and progesterone receptors and HER2 (triple markers), with the intention of malignancy grading. Enhanced expression of calretinin and placental alkaline phosphatase, without immunoreaction to c-Kit in neoplastic cells, is related to high-grade malignancy. Out of 50 tumors, 31 were metastasized, 29 of which (93.5%) were moderately to strongly calretinin positive (P<0.05). However, the results for c-Kit - and Plap+ in metastatic tumors were not reproducible. It may be concluded that calretinin could be introduced as a determinant biomarker in the diagnosis of breast cancer metastasis.

Key words: biomarker; canine breast cancer; calretinin; c-kit; placenta alkaline phosphatase

Introduction

It has been well established that estrogen receptor (ER) expression, along with progesterone receptor (PR) and epidermal growth factor receptor 2 (HER2), which are known as the triple markers, are prognostic and predictive in evaluation and management of malignant canine mammary tumors (CMTs) and human breast cancer (ONITIL et al., 2009).

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Nevertheless, some tumors have no response to any of these immunohistochemical markers, and therefore, employing other markers to unveil concealed cancer profiles is unavoidable (TALIANO et al., 2013).

In light of recent studies, many biomarkers have frequently been used to assess the tumor-grading score as a novel standard.

In the present research, three immunomarkers, including c-Kit (CD117), calretinin, and placental alkaline phosphatase (Plap) were used on tissue samples, to be compared with the triple markers that may be considered as practical tools in delineation of cutoff values between normal, benign, and malignant conditions.

With functions including proliferation, migration, and survival of hematopoietic stem cells, melanoblasts, primordial germ cells, endothelial, epithelial and endocrine cells, c-Kit proto-oncogene may be expressed in different tissues. It is postulated that the c-Kit gene plays a mysterious role in several diseases, with gain-of-function mutations including mastocytoma, gastrointestinal stromal tumors, and glial tumors, besides the loss of function and mutations associated with breast cancer (BERNEX et al., 1996; MAFFINI et al., 2008; MAHZOUNI and JAFARI, 2012).

Calretinin is a 29-KD calcium binding protein, primarily expressed in neuronal cells. However, it can be tracked in other normal or neoplastic cells (LUGLI et al., 2003; ROGERS 1987; TALIANO et al., 2013). The main application of this protein is to distinguish mesothelioma from poorly differentiated pulmonary adenocarcinomas, but this marker may also be expressed in other malignant tumors affecting ovaries, testes, adrenal glands, the colon, breasts, the thymus, skin, and soft tissue (LUGLI et al., 2003; POWELL et al., 2011; TALIANO et al., 2013).

Located in certain polarized epithelial cell types, placental alkaline phosphatases are glycosyl phosphatidylinositol-lipid anchored membrane proteins, with wide substrate specificity (FISHMAN 1987, HARRIS 1990), producing an organic radical and inorganic phosphate in an alkaline medium as a catalyzer, by hydrolysis of phosphate esters (REICHLING and KAPLAN, 1988). Several isoenzymes of ALP are detected in specific organs. These enzymes are derived from the liver, bones, kidneys, placenta, intestines, and leukocytes (FRIEDMAN et al., 1996).

Diagnosis of a variety of diseases is related to serum ALP elevation, and it is a reliable and specific marker for some complications, including biliary cirrhosis, sclerosing cholangitis, hepatic lymphoma, breast cancer, and sarcoidosis (NEUSCHWANDER-TETRI, 1995).

Although remarkable achievements, such as molecular assays, are characterized as high standards for classification of breast cancer (for example, c-Kit is a receptor tyrosine kinase (RTK) encoded by the KIT gene, and its mutations have recently been evaluated (KOLTAI et al., 2018) or in an earlier study (KUBO et al., 1998), as traditional test), Immunohistochemistry (IHC) is more available and reliable than other methods (PARISE and CAGGIANO, 2014).

Materials and methods

Sample size.
Fifty formalin fixed samples of primarily surgically excised malignant mammary tumors were collected from dogs, hospitalized at the Small Animal Teaching Hospital, University of Tehran, private veterinary hospitals and the veterinary clinic of Lorestan University. Of these, 31 tumors had metastasized to the regional lymph nodes or distant organs, as diagnosed by FNA, imaging techniques, surgery, and in necropsy findings (Table 1) and the malignancy of the remainder was confirmed according to histopathological profiles (therefore, all were grade 3). Moreover, 29 of the tissue specimens were identified as malignant (including 17 metastatic and 12 non-metastatic specimens) by the auxiliary method of MALDI-TOF, mass spectrometry prior to histopathological evaluation (ZAMANI-AHMADMAHMUDI et al., 2014).

Histopathology. The histopathological typing and grading of malignant neoplasms in the current research were carefully classified according to the new classification system proposed by GOLDSCHMIDT et al., 2011, depicted in Table 1. It is noteworthy that several cases were found to have two or more histological patterns.
Table 1. Histopathological pattern and score staining of immunomarkers in patients

| No. | Pattern                                                                 | Er  | Pr  | Her-2 | Cal | c-Kit | Plap | Metastasis           |
|-----|-------------------------------------------------------------------------|-----|-----|-------|-----|-------|------|---------------------|
| 1   | Papillary cystic + ductal carcinoma + anaplastic carcinoma              | 0   | 0   | 1     | 2   | 0     | 1    | LN, Liver           |
| 2   | Solid carcinoma                                                         | 0   | 0   | 0     | 2   | 0     | 2    | LN                 |
| 3   | Tubular - tubulopapillary + papillary cystic carcinoma                  | 0   | 0   | 0     | 3   | 0     | 1    | LN                 |
| 4   | Ductal carcinoma                                                        | 0   | 0   | 0     | 1   | 0     | 0    | ND                 |
| 5   | Anaplastic carcinoma                                                    | 0   | 0   | 0     | 0   | 0     | 0    | -                  |
| 6   | Complex carcinoma                                                       | 0   | 0   | 0     | 3   | 1     | 1    | Lung               |
| 7   | Tubular carcinoma                                                       | 0   | 0   | 0     | 2   | 0     | 0    | LN, Lung           |
| 8   | Tubular carcinoma with interlobular fibrosis                            | 0   | 0   | 0     | 2   | 0     | 0    | Lung, Spleen       |
| 9   | Tubular carcinoma                                                       | 0   | 0   | 0     | 2   | 0     | 0    | LN, Lung           |
| 10  | Carcinoma and malignant myoepithelioma                                  | 0   | 0   | 2     | 2   | 1     | 2    | LN, Lung           |
| 11  | Tubular carcinoma                                                       | 0   | 0   | 0     | 1   | 0     | 0    | -                  |
| 12  | Carcinosarcoma + carcinoma and malignant epithelioma + inflammatory carcinoma | 1   | 0   | 0     | 3   | 1     | 0    | LN                 |
| 13  | Tubulopapillary carcinoma                                               | 0   | 0   | 1     | 1   | 0     | 0    | -                  |
| 14  | Carcinoma in situ                                                       | 0   | 2   | 0     | 2   | 2     | 0    | -                  |
| 15  | Solid carcinoma                                                         | 0   | 1   | 0     | 3   | 0     | 0    | LN                 |
| 16  | Carcinosarcoma                                                          | 0   | 0   | 0     | 0   | 0     | 0    | LN                 |
| 17  | Tubulo - micropapillary, intraductal carcinoma + lipid laden            | 0   | 0   | 0     | 3   | 0     | 0    | LN, Lung           |
| 18  | Complex carcinoma                                                       | 0   | 0   | 0     | 0   | 0     | 0    | -                  |
| 19  | Complex carcinoma                                                       | 0   | 0   | 0     | 3   | 0     | 0    | LN                 |
| 20  | Complex carcinoma                                                       | 2   | 1   | 2     | 3   | 0     | 0    | Lung               |
| 21  | Intraductal papillary carcinoma to tubulopapillary carcinoma            | 0   | 0   | 3     | 3   | 0     | 1    | Lung               |
| 22  | Microcellular carcinoma + intraductal papillary carcinoma + solid adenocarcinoma | 0   | 1   | 3     | 3   | 0     | 0    | LN, Lung           |
| 23  | Carcinosarcoma + tubular carcinoma                                      | 2   | 0   | 3     | 3   | 0     | 1    | Liver              |
| 24  | Tubulopapillary +cribriform                                            | 0   | 0   | 0     | 0   | 2     | 2    | ND                 |
| 25  | Cribriform carcinoma                                                    | 0   | 0   | 0     | 3   | 1     | 0    | LN                 |
| 26  | Solid carcinoma                                                         | 0   | 0   | 1     | 0   | 0     | 0    | -                  |
| 27  | Tubular carcinoma                                                       | 0   | 0   | 0     | 2   | 0     | 1    | LN                 |
| 28  | Intraductal carcinoma                                                   | 0   | 0   | 3     | 0   | 0     | 0    | -                  |
| 29  | Tubulopapillary carcinoma + anaplastic carcinoma                        | 3   | 0   | 2     | 2   | 1     | 1    | -                  |
| 30  | Tubular carcinoma                                                       | 0   | 0   | 0     | 3   | 0     | 0    | LN                 |
| 31  | Intraductal carcinoma                                                   | 0   | 0   | 0     | 0   | 0     | 2    | LN, Bone           |
| 32  | Carcinosarcoma                                                          | 0   | 0   | 2     | 1   | 0     | 0    | -                  |
Table 1. Histopathological pattern and score staining of immunomarkers in patients (continued)

| No. | Pattern                          | Er  | Pr  | Her-2 | Cal  | c-Kit | Plap | Metastasis |
|-----|----------------------------------|-----|-----|-------|------|-------|------|------------|
| 33  | Tubular carcinoma                | 0   | 0   | 3     | 3    | 0     | 1    | Lung       |
| 34  | Tubular carcinoma                | 0   | 0   | 0     | 1    | 2     | 2    | -          |
| 35  | Complex carcinoma                | 0   | 0   | 0     | 1    | 0     | 0    | -          |
| 36  | Tubular carcinoma                | 0   | 0   | 0     | 2    | 0     | 1    | LN, Lung   |
| 37  | Solid + intraductal carcinoma    | 0   | 0   | 0     | 0    | 0     | 0    | -          |
| 38  | Solid + cribriform carcinoma     | 2   | 3   | 2     | 0    | 0     | 2    | ND         |
| 39  | Tubular carcinoma                | 0   | 0   | 3     | 2    | 2     | 1    | LN, Lung   |
| 40  | Complex carcinoma                | 0   | 0   | 3     | 2    | 0     | 2    | LN         |
| 41  | Intraductal carcinoma            | 0   | 0   | 2     | 1    | 0     | 0    | -          |
| 42  | Solid carcinoma                  | 2   | 2   | 3     | 0    | 0     | 1    | -          |
| 43  | Tubulopapillary carcinoma        | 2   | 2   | 3     | 3    | 0     | 0    | LN, Lung   |
| 44  | Complex carcinoma                | 2   | 0   | 3     | 3    | 0     | 1    | LN         |
| 45  | Complex carcinoma                | 0   | 0   | 3     | 3    | 0     | 0    | LN         |
| 46  | Anaplastic carcinoma             | 0   | 0   | 3     | 2    | 0     | 0    | Bone       |
| 47  | Tubular + Cribriform carcinoma   | 0   | 0   | 3     | 3    | 0     | 1    | LN         |
| 48  | Tubulopapillary carcinoma        | 0   | 0   | 3     | 3    | 2     | 0    | LN, Lung   |
| 49  | Tubulopapillary + Micropapillary | 0   | 0   | 3     | 1    | 2     | 2    | -          |
| 50  | Cribriform to comedocarcinoma    | 0   | 0   | 3     | 1    | 3     | 1    | -          |

Er, estrogen; Pr, progesterone; Her-2, human epidermal growth factor receptor-2; Cal, calretinin; Plap, placental alkaline phosphatase; ND, not defined; LN, lymph node

Table 2. Antibody specifications and pattern of staining

| Antibody               | Clone        | Dilution | Time of incubation | Pattern of staining          |
|------------------------|--------------|----------|--------------------|-------------------------------|
| ER monoclonal          | 1D5          | 1:100    | 80 min             | Nuclear                       |
| PR monoclonal          | PgR 636      | 1:100    | 80 min             | Nuclear                       |
| HER2 monoclonal        | PN2A         | 1:100    | overnight at 4 °C  | Membranous                    |
| c-Kit polyclonal       | A4502 Catalog no | 1:500 | 80 min             | Cytoplasmic with membranous accentuation |
| Calretinin monoclonal  | DAK-Calret1  | 1:50     | overnight at 4 °C  | Nuclear, cytoplasmic          |
| PLAP monoclonal        | 8A9          | 1:50     | 80 min             | Membranous cytoplasmic        |

Immunohistochemical procedure. Paraffin-embedded blocks of each tumor were used to prepare 4 µm-thick consecutive sections. The sections were then deparaffinized in xylene and rehydrated. Endogenous peroxidase was blocked by immersion in 0.3% H2O2 for 20 minutes. For antigen retrieval, the slides were incubated with Tris-EDTA (pH=9) for 15 minutes in an oven. In the next step, the slides were covered by primary antibodies for ER (clone ID5, Dako, Glostrup, Denmark, Cat No. M7047) at a dilution of 1:100, PR (clone PgR 636; Dako; M3569 1:100) HER2-pY-1248 (Phosphorylation site specific) (clone PN2A, Dako; 1:100), c-Kit (A4502, Dako; 1:500), Calretinin (clone DAK-Calret1, Dako; M7245, 1:50) and Plap (clone 8A9, Dako; MA5-14064, 1:50) followed by incubation with a Dako REAL™ EnVision Detection System K5007. All the slides were then covered by 0.05% diaminobenzidine dihydrochloride solution (DAB), used as a chromogen, for 10 minutes, at ambient temperature. Antibody specifications and staining patterns are presented in Table 2.
Control samples. Positive and negative controls were included whenever possible to monitor the correct performance of the processed tissues. Positive internal control samples were selected from tumor blocks containing normal and non-neoplastic tissue. The external positive control samples are depicted in Table 3. For negative controls, homologous nonimmune serum or buffer replaced the primary antibodies.

Scoring and statistical analysis. The scoring system was almost the same as the Quick score by PENA et al. (2014).

It was modified for the percentage of cells labeled (no labeling = −/+, labeling ≤10% = +, 10 up to 50% = ++, and more than 50% = +++), the score for intensity (absent = −, weak =+, moderate =++, and strong = +++). Only 2+ and 3+ for HER2 overexpression were determined as criteria, and +1 was excluded on the basis of the description by ORDAS et al. (2007).

Statistical analyses were performed by SPSS Version 19.0. The data were described as absolute and relative frequencies. The data were analyzed by Chi-square and Fisher’s exact test. A P-value ≤0.05 was considered statistically significant.

| No | Pattern           | Er | Pr | Her-2 | Cal | c-Kit | Plap |
|----|-------------------|----|----|-------|-----|-------|------|
| 1  | Normal            | 2  | 2  | 0     | 0   | 3     | 0    |
| 2  | Normal            | 2  | 1  | 0     | 0   | 3     | 0    |
| 3  | Normal            | 3  | 2  | 0     | 0   | 2     | 0    |
| 4  | Normal            | 2  | 0  | 0     | 0   | 2     | 0    |
| 5  | Normal            | 1  | 2  | 0     | 0   | 3     | 0    |
| 6  | Solid adenoma     | 0  | 0  | 2     | 1   | 1     | 0    |
| 7  | Complex adenoma   | 0  | 0  | 3     | 0   | 0     | 0    |
| 8  | Lobular hyperplasia | 0 | 0  | 2     | 0   | 1     | 0    |
| 9  | Fibroadenoma      | 0  | 0  | 3     | 0   | 0     | 0    |
| 10 | Benign mixed      | 0  | 0  | 2     | 0   | 0     | 0    |
| 11 | Lobular hyperplasia | 1 | 0  | 3     | 0   | 1     | 0    |
| 12 | Ductal adenoma    | 0  | 0  | 2     | 1   | 0     | 0    |

Er, estrogen; Pr, progesterone; Her-2, human epidermal growth factor receptor-2; Cal, calretinin; Plap, placental alkaline phosphatase.

Results

The results of immunohistochemical analysis are presented in Table 1. Nuclear immuno-staining of epithelial cells and to a lesser extent, in stromal cells, were shown for ER and PR in normal samples. HER2 had positive membranous immunolabeling in tumor cells.

Calretinin positive cells were shown in luminal type tumor structures as linear patterns, in which the immunolabeled cells with both cytoplasmic and nuclear immunooexpression were located in the innermost layer adjacent to the basement membrane. However, few myoepithelial cells reacted to this marker (Fig. 1; A, B). Conversely, widespread intermingled staining of neoplastic cells in other tumor types did not follow the specified pattern (Fig. 1; C-F). Calretinin expression was negative in all normal and most benign breast tumors, and weakly expressed in the rest (Table 3). Diffuse and strong cytoplasmic immunostaining by c-Kit was observed in normal epithelium (Fig. 2; A, B), while focal cytoplasmic and membranous expression was defined in benign or malignant neoplastic cells. A similar staining pattern was also determined in tumor cells by Plap, with no positively stained cells in the control group (Fig. 3; A-D).
Fig. 1. Calretinin immunostaining. 1A - Tubular carcinoma, dog No. 23. Linear pattern; 1B - Higher magnification of Fig. 1A; Nuclear and cytoplasmic immunopositive in epithelial neoplastic cells; 1C - Complex carcinoma, dog No. 23. Diffuse immunostaining; 1D - Higher magnification of Fig. C. Both nuclear and cytoplasmic immunolabeling; 1E - Cribriform carcinoma, dog No. 25. Diffuse immunolabeling; 1F - Higher magnification of Fig.1 E. Both nuclear and cytoplasmic immunoexpression.
Fig. 2. c-Kit immunostaining. Control mammary gland; 2A - Diffuse cytoplasmic immunolabeling; 2B - Higher magnification of Fig. 2A.

Fig. 3. Plap immunostaining. 3A - Cribriform-comedocarcinoma, dog No. 50. Cytoplasmic to membranous immunolabeling; 3B - Higher magnification of Fig. 3A; 3C - Solid carcinoma, dog No. 2; 3D. Higher magnification of Fig. 3C.
The differences between calretinin+/metastasis and calretinin+/c-Kit -/Plap+/metastasis were statistically significant (P<0.05); however, there were no statistical differences between ER-/calretinin+/metastasis, c-Kit -/metastasis and Plap+/metastasis (P>0.05).

Also, authentication of calretinin was employed in five canine metastasized tissue samples, including two regional lymph nodes (case no. 30 and 47), two pulmonary, and one liver specimen, in which the neoplastic cells responded as they did in mammary tumors.

Table 4 highlights the correlation of triple markers with calretinin, c-Kit, and Plap, as well as the possible contribution of each biomarker in malignant and metastatic breast cancers.

Table 4. Evaluation of immunohistochemical expression of triple markers, calretinin, c-Kit and Plap in metastatic patients

| Group | Cal+ | c-Kit+ | Plap+ | Total |
|-------|------|--------|-------|-------|
| ER/PR/HER2- | 5 (16.1) | | | |
| ER/PR/HER2- | 1 (3.2) | | | |
| ER/PR/HER2- | 6 (19.3) | | | |
| ER/PR/HER2- | 2 (6.4) | | | |
| ER/PR/HER2- | 1 (3.2) | | | |
| ER/PR/HER2- | 2 (6.4) | | | |
| ER/PR/HER2- | 4 (12.9) | | | |
| ER/PR/HER2- | 2 (6.4) | | | |
| ER/PR/HER2- | 1 (3.2) | | | |
| ER/PR/HER2- | 1 (3.2) | | | |
| ER/PR/HER2- | 2 (6.4) | | | |
| ER/PR/HER2- | 2 (6.4) | | | |
| ER/PR/HER2- | 1 (3.2) | | | |
| ER/PR/HER2- | 2 (6.4) | | | |
| ER/PR/HER2- | 31 (100) | | | |

With regard to Table 5, it may be understood that the highest number of metastases were in the groups of triple negative 16/31 (51.6%) and ER/PR, HER2- 8/31 (25.8%).

Table 5. The different states of triple markers (ER, PR, and HER2) and their correlation with calretinin in mammary gland metastases

| Triple groups | Cal+ | Cal- | Total |
|---------------|------|------|-------|
| ER/PR/HER2-   | 14 (45.1) | 2 (6.4) | 16 (51.6) |
| ER/PR/HER2-   | 8 (25.8) | 0 (0) | 8 (25.8) |
| Others        | 7 (22.5) | 0 (0) | 7 (22.5) |
| Total         | 29 (93.5) | 2 (6.4) | 31 (100) |

Discussion

Alongside using triple markers, it might be necessary to optimize the exploitation of other markers in screening for and diagnosing breast cancer.

Recently, tumor markers have been developed in breast cancer research, due to their impact on prognosis, treatment, survival, and for their relationship to breast cancer subtypes (BAUER et al., 2007).

Determination of both ER and HER2 status in breast cancer is useful as prognostic and predictive factors, and this has become standard practice in the management of breast tumor (BAUER et al., 2007). HER2 positivity has a direct link with aggressive tumor behaviors (PENA et al., 2014), while PR is only prognostic (HEFTI et al., 2013). The importance of canine HER2 overexpression in breast tumors remains unclear; although some researchers believe that in dogs too HER2 expression provides malignant conditions in breast tissue, but the average rate of its expression is a little above 30% in malignant tumors, and therefore it is not recognized as a reliable marker (AHREN et al., 1996; PENA et al., 2014). As shown in Table 2, apart from normal breast tissues that were HER2 negative, all benign tumors were moderately to strongly HER2 positive. RUNGSIPIPAT et al. (1999) in their study showed that compared to 19.1% of malignant tumors, half of 32 benign tumors were HER2 positive, therefore, its expression is higher in benign lesions than in malignant ones. Thus, a lower expression rate of HER2 is more probably associated with an unfavorable prognosis.
The results from medical literature studies reveal that unlike in humans, hormone receptor levels are significantly lower in canine breast cancer, meaning that hormone independency is more apparent in dogs (RICKETTS et al., 1991; RUTTEMAN et al., 1988). It was concluded that canine ER and PR positivity were regularly lower than in normal breast tissue or benign lesions (RUTTEMAN et al., 1988). Therefore, it seems that elevation of ER and PR is directly related to pathological cell proliferation, and the expression of ER in normal human breast is favorable to breast cancer development (RICKETTS et al., 1991). Nevertheless, whereas in normal breast tissue and benign tumors, they are constant, in malignancy, canine ER and PR levels vary and tend to fall (RICKETTS et al., 1991; RUTTEMAN et al., 1988). While the expression of ER and PR in our normal dogs were consistent with literature, however lack of expressions were in conflict in benign tumors except one (case No.11, Table 3).

PR receptor expression takes place in almost two-thirds of carcinomas (CHANG et al., 2009; GERALDS et al., 2000; GUIL-LUNA et al., 2014; MARTIN de LAS MULAS et al., 2005), where a simple epithelial-type carcinoma, with the lowest levels of expression, this sticking point covers all benign tumors (GUIL-LUNA et al., 2014). Simple epithelial-type carcinomas display less insensible PR expression compared to complex or mixed subtypes, and there is an association between PR expression and malignancy of tumors (GUIL-LUNA et al., 2014; MILLANTA et al., 2005). As in our results, PR was not expressed in most exclusively ER- (38/50; 76%) lesions defined as having worse prognosis; however, all these are unlike the lowest expression of PR in experiments even in well-differentiated tumors (MOUSER et al., 2010).

However, for more accurate determination of prognosis and survival rate, usually both ER/PR and triple markers have been sporadically analyzed together, as follows: ER+/PR+, HER2- is the most prevalent subtype, ER+/PR+; HER2+ has good prognosis, while, both ER- PR-, HER2+ and triple negative (ER/PR-, HER2-) usually have a poor response to chemo-drugs, with a reduction in life expectancy (ONITIL et al., 2009).

Although calretinin is a particular biomarker of mesothelial cells broadly employed for malignant mesothelioma recognition, its expression is demonstrated in carcinomas, such as malignant breast tumors and sarcomas (LUGLI et al., 2003; TALIANO et al., 2013). Lung, salivary gland, ovary, stomach, and colon carcinomas have been studied for this marker (POWELL et al., 2011). Moreover, calretinin is a promising candidate for testicular tumor diagnosis (CAO et al., 2001).

In veterinary literature, this marker has been applied in normal and neoplastic canine testicles. Only Leydig and Sertoli cells reacted to calretinin in terms of its response to conventional drugs. There is verified documentary evidence that PR is controlled by ER; therefore, ER- is a measure for PR-. Furthermore, PR expression has no logical connection with ER in the prognosis of breast cancers, and most authors believe that ER-/PR+ is a false subtype, although it remains controversial (HEFTI et al., 2013). ER-/PR+, in different literature, accounts for between zero and seven percent of breast cancer (BARDOU et al., 2003; COLDITZ et al., 2004; DE MAEYER et al., 2008; HEFTI et al., 2013; NADJI et al., 2005). It must be counted as a distinct subtype, showing clinicopathological signs, and so has worse prognosis than both ER+/PR+ and ER+/PR-, but better than an ER-/PR-patient. Nevertheless, the contingency of clinical signs is more similar to ER+ than ER-/PR- (CHAN et al., 2015; DE MAEYER et al. 2008; SHEN et al., 2015). However, 3/50 (6%) of our tumors appeared as ER-/PR+, all of which were Calretinin positive, and although the PR stained weakly, we could not rule these out as an artifact after a repeated test for both ER and PR, which had the same results. Perhaps, as in humans, it meant that the dogs were juvenile or had a higher tumor grade profile (CHAN et al., 2015; DE MAEYER et al., 2008).

If breast cancers are classified according to the triple markers, they provide valuable evidence for treatment policy. As a standard source, ER+/PR+, HER2- is the most prevalent subtype, ER+/PR+; HER2+ has good prognosis, while, both ER- PR-, HER2+ and triple negative (ER/PR-, HER2-) usually have a poor response to chemo-drugs, with a reduction in life expectancy (ONITIL et al., 2009).
normal testis; however, its expression was revealed in all varieties of testicular tumors on the basis of the priority order, including Leydig cell tumor, seminoma, and Sertoli cell tumor (Ciaputa et al., 2014; Radi and Miller 2005).

Canine oral lesions such as acanthomatous ameloblastoma (CAA) and oral squamous cell carcinoma (OSCC) has also been tested with calretinin, with a significant reaction of the OSCC (Fulton et al., 2014).

A strong correlation between calretinin expression and metastasis was found first in this study (P<0.05). It is anticipated that calretinin plays an important role in epithelial-mesenchymal transition (EMT); therefore, with vimentin expression, these markers might be considered as metastatic potentiation indicators (Hennessy et al., 2009; Taliano et al., 2013; Zhang et al., 2012).

Well-differentiated and individual ER+ tumors are generally calretinin negative, and, conversely, tumors with stronger calretinin expression are high grade. ER- is often associated with poor prognosis (Lugli et al., 2003; Powell et al., 2011; Taliano et al., 2012).

Currently, only a few sources are available in the veterinary literature about the role of Plap and its diagnostic value in diseases. For example, its elevation was noticed as a good indicator in some canine conditions such as hepatic and endocrine diseases, and osteopathy (Milne 1985), but it has not been fully investigated in neoplasms.

The expression of placental alkaline phosphatase was directly associated with some types of cancers in human (Chang et al., 1994; Fishman 1987; Lange et al., 1982) and its function was induced by progestin in ER+ breast cancer cell lines, and by estrogen in endometrial adenocarcinoma cells (Chang et al., 2009; Di Lorenzo et al., 1993; Di Lorenzo et al., 1991). Our results in this context are significantly different. According to Table 1, only 2/22 (9%) of cases followed this pattern (ER+/PR+ Plap+) and 3/22 (13.6%) were (ER+/PR- Plap+) while 17/22 (77%) were (ER-/PR- Plap+) and Plap function might be affected by other factors apart from ER/PR. The production of this tumor marker is associated with an oncodevelopmental gene, sometimes detectable in low levels of normal human serum, but excessive expression is indicative of cancer (Mossner et al., 1984). In accordance with the literature data, Plap expression is demonstrated in 20% of breast carcinomas (22/50 [44%] in present data) and in 75% of intestinal carcinomas, and is not traceable in normal breast tissue or benign tumors (Mossner et al., 1984).

Therefore, Plap might be considered a potential biomarker in the diagnosis of malignant breast tumors, exclusively on fixed tissue sections using the immunohistochemical method (Mcdicken et al., 1983; Mossner et al., 1984; Stigbrand et al., 1985).

Plap was not recognized in this study in either normal or benign tumors, although it appeared in 22 malignant cases, 15 of which were metastasized. Similar to others, it is not beyond possibility that Plap induction has a direct link with malignancy.

In normal epithelial breast cells, the c-Kit receptor is located in large amounts on the membrane or cytoplasm of ductal or alveolar structures (Chui et al., 1996; Maffini et al., 2008), but with cellular proliferation in benign lesions, its amount is impressively reduced and it is absolutely unrecognizable in poorly differentiated cells (Maffini et al., 1992; Natali et al., 1992; Ulivi et al., 2004). As shown in Table 2, our results are fully in line with those of other researchers. C-Kit was decisively expressed in normal breast tissues and gradually reduced or completely absent in benign tumors. The evidence indicates that homoeostasis regulation and differentiation of normal mammary epithelial cells are taken up by c-Kit in a complex molecular network, and it achieves second place in maintenance of normal growth in glandular epithelial cells after β1 integrin (Regan et al., 2012). Therefore, in the absence of c-Kit, malignancy process could develop (Maffini et al., 2008; Natali et al., 1992). One of the original mechanisms that contributes to c-Kit receptor loss in normal cells and subsequently accumulation of malignancy characteristics, is probably related to tyrosine kinase inhibitors and
the reduction in c-Kit activity (MAFFINI et al., 2008; SAMOSZUK and CORWIN 2003).

Surprisingly, in their study on canine breast cancer using real-time RT-PCR, KOLTAI et al. (2018) found that c-Kit mutation occurred in 13 dogs with mammary gland carcinoma, measured with other tyrosine kinases markers (including; VEGF, VEGFR1, PDGFR1, EGFR and c-MET), and concluded that mRNA expression of c-Kit was significantly higher than in normal breasts. In a similar work on 11 dogs, c-Kit RT-PCR product was higher in all benign and malignant carcinomas; however, its expression was low in normal breasts (KUBO et al., 1998).

This is inconsistent with the present study and other results. The discrepancy might be related to the methodology. For example, ULIVI et al. (2004) studied c-Kit and its ligand (SCF) in normal cells, in situ, and invasive breast cancers using immunohistochemistry for c-kit and SCF protein, and an in situ hybridization technique, as well as RT-PCR for mRNA expression. Both protein and mRNA expression of c-Kit and SCF were high in normal glands, lower in in situ, and absent in invasive breast cancer in IHC and in situ hybridization. However, by RT-PCR mRNA expression, they were displayed in both normal and breast tumors.

The authors believed that the variable results were due to the application of different methodologies, and concluded that morphological methods are the most reliable and applicable for detection of c-Kit and SCF in normal and tumor cells. In another study, c-Kit was evaluated in normal, non-metastatic, and metastatic breast cancer on the basis of its pattern as precursor of canine mast cell tumor (MCT) (SALVADOR et al., 2013). The results were unexpected and not in accordance with the (MCT) profile pattern. However, they had not pointed out score and the protein was expressed in all samples.

In our results, 30.7% and 50% of normal internal glands were calretinin positive in those tumors with +2 and +3 intensity scoring, respectively (p=0.3) using the independent t-test. It was 37.5% for Plap with intensity 2 and 100% negative for c-Kit, and score zero. Perhaps this means that upregulation of calretinin and Plap in some normal glands precedes or is concurrent with its expression in neoplastic epithelial cells, whereas gene knockout is uniformed in both normal and neoplastic cells for c-Kit. Therefore, it must be assumed that it is inappropriate to consider normal glands alongside malignant structures as an internal control, and for proper evaluation, we strongly recommend using normal external controls.

**Conclusion**

Calretinin could be very useful alone or in conjunction with ER, PR, and HER2 antibodies in identifying and prognosis of malignant mammary gland tumors, from normal or benign lesions. However, another marker such as c-Kit and Plap can also play a potential role in defining such differences. Although the ER- was not significant in metastatic tumors, it must still be considered that 24/31 of metastatic tumors (77.4%) were in the ER-/PR-/HER2+/- group which is not insignificant.

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

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SAŽETAK
Maligni tumori dojke najčešći su tumori u ljudi i povezani su s lošom prognozom. Da bi se testirali novi dijagnostički postupci i terapijske procedure u ljudi, potreban je prikladan životinjski model tumorogeneze mliječne žlijezde. Psi su potencijalno dobar model zbog spontanog razvoja ovakvih tumora. U ovom su istraživanju, s ciljem stupnjevanja malignosti, međusobno uspoređena tri imunomarkera, kalretinin, c-Kit (CD117) i placentalna alkalna fosfataza (Plap), a zatim su isti uspoređeni i s estrogenskim, progesteronskim te HER2 (trostrukim) markerima. Povećanje izražajnosti kalretinina i placentalne alkalne fosfataze, bez imunoreakcije na c-Kit u neoplastičnim stanicama povezano je s visokim stupnjem malignosti. Od 50 tumora, 31 je metastazirao, od kojih je 29 (93,5 %) bilo umjereno do izrazito pozitivno na kalretinin (P < 0,05). Doduše, rezultati za c-Ki ti Plap+ nisu bili ponovljivi. Zaključujemo da bi kalretinin mogao poslužiti kao biomarker u dijagnostici metastatskog raka dojke.

Ključne riječi: biomarker; tumor mliječne žlijezde u pasa; kalretinin; c-kit; placentalna alkalna fosfataza

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