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Immunoexpression of metalloproteinases 9 (MMP-9) and 2 (MMP-2) and their inhibitors (TIMP-1 and TIMP-2) in normal and neoplastic canine mammary tissue

Imunomarcação de metaloproteinases 9 (MMP-9) e 2 (MMP-2) e seus inibidores (TIMP-1) e (TIMP-2) no tecido mamário canino normal e neoplásico

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Abstract
The aim of this study was to perform the immunostaining of MMP-9 and MMP-2 and its inhibitors, TIMP-1 and TIMP-2, on normal and neoplastic canine mammary tissue in order to evaluate the behavior of these proteins in extracellular matrix (ECM) remodeling in different neoplastic mammary types. Thus, 48 samples of canine mammary tissue were analyzed, 14 of which complex carcinomas, 13 tubulopapillary carcinomas, six single adenomas and 15 normal mammary tissue. There were differences in MMP-9, TIMP-1 and TIMP-2 according to mammary histomorphology, and MMP-9 presented increased immunoexpression in epithelial and stromal cells in tubulopapillary and complex carcinomas. TIMP-1 exhibited reduced immunostaining in the stromal cells of the complex carcinomas and TIMP-2 enhanced immunostaining in the epithelial cells of tubulopapillary carcinomas. There was a positive correlation between MMP-9 and TIMP-1 in epithelial and stromal cells regarding immunostaining intensity and number of labeled cells in the normal breast. There was a positive correlation between MMP-9 and TIMP-2 in the epithelial cells of tubulopapillary carcinomas. It is concluded that balanced activity between MMP-9, MMP-2, TIMP-1 and TIMP-2 maintains normal canine mammary tissue homeostasis while increased immunoexpression of MMP-9 and TIMP-2 and reduced TIMP- 1 in carcinomas suggest a favorable condition for tumor evolution.
Keywords: dog, mammary gland, neoplasm, matrix metalloproteinases.

Resumo
Este estudo teve como objetivo realizar a imunomarcação de MMP-9 e MMP-2 e seus inibidores, TIMP-1 e TIMP-2, no tecido mamário canino normal e neoplásico, a fim de avaliar o comportamento dessas proteínas no remodelamento da matriz extracelular (MEC) em diferentes tipos neoplásicos mamários. Foram analisadas 48 amostras de tecido mamário canino, sendo 14 carcinomas complexos, 13 carcinomas

tubulopapilares, seis de adenomas simples e 15 mamas sem alterações. Houve diferença em MMP-9, TIMP-1 e TIMP-2 de acordo com a histomorfologia mamária, sendo que MMP-9 apresentou maior imunoexpressão em células epiteliais e estromais em carcinomas tubulopapilares e complexos. TIMP-1 exibiu menor imunomarcação nas células estromais dos carcinomas complexos e TIMP-2 maior imunomarcação nas células epiteliais dos carcinomas tubulopapilares. Houve correlação positiva entre MMP-9 e TIMP-1 nas células epiteliais e estromais quanto à intensidade de imunomarcação e número de células marcadas na mama normal. Houve correlação positiva entre MMP-9 e TIMP-2 nas células epiteliais dos carcinomas tubulopapilares. Conclui-se que a atividade equilibrada entre MMP-9, MMP-2, TIMP-1 e TIMP-2 mantém a homeostase do tecido mamário canino normal enquanto a imunoexpressão aumentada de MMP-9 e TIMP-2 e a imunoexpressão reduzida de TIMP-1 nos carcinomas sugere condição propícia à evolução tumoral.
Palavras-chave: cão, glândula mamária, neoplasia, metaloproteinases de matriz.

Introduction

Mammary neoplasms can represent 50% to 70% of tumors diagnosed in intact bitches[1], and 20% to 80% of these cases are classified as malignant when submitted to histopathological examination[2].

Malignant tumors have metastatic potential. However, for this to occur, neoplastic cells must detach themselves from the primary tumor mass, cross physical barriers such as the extracellular matrix (ECM), reach a distant tissue, and proliferate there[3-5]. Bearing in mind that the degradation of the basement membrane (BM) and ECM comprises one of the initial events of the metastatic process[6, 7], matrix metalloproteinases (MMP) have been studied due to their ability to degrade components of BB and ECM[8], culminating in tumor progression and metastasis[9, 10]. In this context, knowledge of MMP is also important to guide better forms of treatment for different types of cancer[11, 12].

MMPs are proteolytic enzymes that act in physiological and pathological processes[13] and secreted in the extracellular medium as inactive pro-enzymes, which are cleaved or conjugated to other components to produce the active enzyme[14, 15]. MMP activity is preferably controlled by tissue matrix metalloproteinase inhibitors (TIMP), which can be of four types (TIMP-1 to TIMP-4) and bind to several MMP, with TIMP-1 and TIMP-2 inhibiting MMP-9 and MMP-2, respectively[16, 17].

In humans, increased immunoexpression of MMP in different tumors, especially MMP-2 and 9, is associated with a high degree of histological undifferentiation, advanced tumor stage, increased risk of metastasis, and death[18-21], which is also observed in breast tumors[18, 22, 23]. On the other hand, the immunoexpression of MMP and its inhibitors in the neoplastic mammary tissue of dogs is still not well understood. However, there has been a description of higher immunostaining of MMP, especially MMP-2 and MMP-9,

and a reduction in TIMP in neoplastic mammary tissue compared to the normal[24, 25]. Aresu *et al.*[26] related MMP-2 and MMP-9 as having an important role in the malignancy of the breast tumor in bitches, but Wu *et al*.[27] concluded that bitches with a high concentration of MMP-9 and low TIMP-1 have a high metastasis rate for lymph nodes, high neoplastic progression, and low overall survival rate.

Therefore, this study aimed to perform the immunostaining of MMP-9, MMP-2, TIMP-1, and TIMP-2 to evaluate the behavior of these proteins in the ECM remodeling in normal and neoplastic canine mammary tissue.

Material and methods

Forty-eight samples of canine mammary tissue were selected from a histopathological diagnosis file, six from simple mammary adenoma (MA), 13 from tubulopapillary carcinoma (TC), and 14 from complex carcinoma (CC), according to the Goldschmidt *et al*.[2] classification, as well as 15 from normal mammary tissue (NM), from bitches with no change in mammary chains.

Two representative cyto-histomorphological fields were also selected from each of the 48 samples for making a tissue microarray (TMA) block and conducting the immunohistochemical study. The preparation of the TMA block followed the technique described by Bubendorf *et al*.[28]. In summary, cylindrical segments (colors) of 2 mm in diameter were removed using a Tissue MicroArray Builder 20010.2 (Histopathology Ltd, Hungary) after identifying representative fields of cyto-histomorphological types in donor blocks and transferred to two receiver blocks (TMA).

Subsequently, 3-µm sections were made from TMA blocks for confirming the selected areas at the HE staining as well as evaluating the MMP-9, MMP-2, TIMP-1, and TIMP-2 immunostaining. The sections for the immunohistochemistry technique were stretched on silanized slides (Starfrost White, Sakura, ready to use, Germany – Dako 9545-1), maintained in an oven at 36 °C for two hours, deparaffinized, hydrated, washed in distilled water and buffer solution TRIS pH 7.4, and incubated in sodium dodecyl sulfate (SDS) solution for 10 minutes.

Then, the cuts were submitted to antigenic recovery, followed by the blocking of endogenous peroxidase in a methanolic solution of 3% H_2O_2 for 15 minutes. Nonspecific reactions were blocked by incubating the slides in a 30% skimmed milk powder (Molico®) solution for 1.5 hours, followed by incubation with anti-MMP-2, anti-MMP-9, anti-TIMP-1, and anti-TIMP-2 antibodies in a humid chamber at 4 °C for 18 hours (Board 1).

The slides were incubated in a signal amplification system (Advance System, Dako K4068) in a humid chamber at room temperature for 30 minutes and the diaminobenzidine solution (DAB, Dako K3468-1) for three minutes to develop the reaction. The buffer solution TRIS pH 7.4 was used to wash the cuts between the steps. It was followed by counterstaining with Harris' hematoxylin for 20 seconds, washing in distilled water, dehydration, clearing, and assembling of slides with coverslips and synthetic resin.

Board 1. Antibodies, dilution, type of antigenic recovery, buffer, and time used in the immunohistochemistry technique

Antibody	Dilution	Antigenic recovery	Buffer	Time
anti-MMP-2 DBS Mob 312	1:25	water bath at 96 °C	Citrate pH 6.0	30 min
anti-MMP-9 Dako A0150	1:200	water bath at 96 °C	TRIS-EDTA pH 9.0	20 min
anti-TIMP-1 Spring E3364	1:100	Pressure chamber* at121 °C	Citrate pH 6.0	30 sec
anti-TIMP-2 Spring E4184	1:150	Pressure chamber* at 121 °C	Citrate pH 6.0	30 sec

*Pascal, DakoCytomation

The immunohistochemical evaluation considered the intensity of immunostaining and the number of labeled cells for MMP-2, MMP-9, TIMP-1, and TIMP-2 in the acinar epithelium and periacinar stroma. The intensity of immunostaining of antibodies was evaluated semi-quantitatively, following the model of Aresu *et al*.[26], using scores from zero to three, with zero being the absence of immunostaining and one, two, and three being slight, moderate, and accentuated immunostaining, respectively. The quantification of acinar epithelial and periacinar stromal cells considered a score zero when there was no immunostaining, and scores one, two, and three when the cells were immunostained in 1–33%, 34–66%, and 67–100% of the tissue, respectively.

The Kruskal-Wallis test was used in the statistical analysis of the variables intensity of immunostaining and the number of immunostained cells relative to the mammary histomorphology, with a significant difference when $p<0.05$. The Spearman correlation test was used to correlate the immunoexpression of antibodies in normal and neoplastic breasts. The data were processed using Excel 2010 and Graphpad Instat version 3.0.

Results

The used antibodies provided cytoplasmic immunostaining, with scores of the intensity of immunostaining and the number of immunostained cells ranging from absent to accentuated, depending on the antibody, cell type, and cyto-histomorphological pattern (Figures 1 and 2).

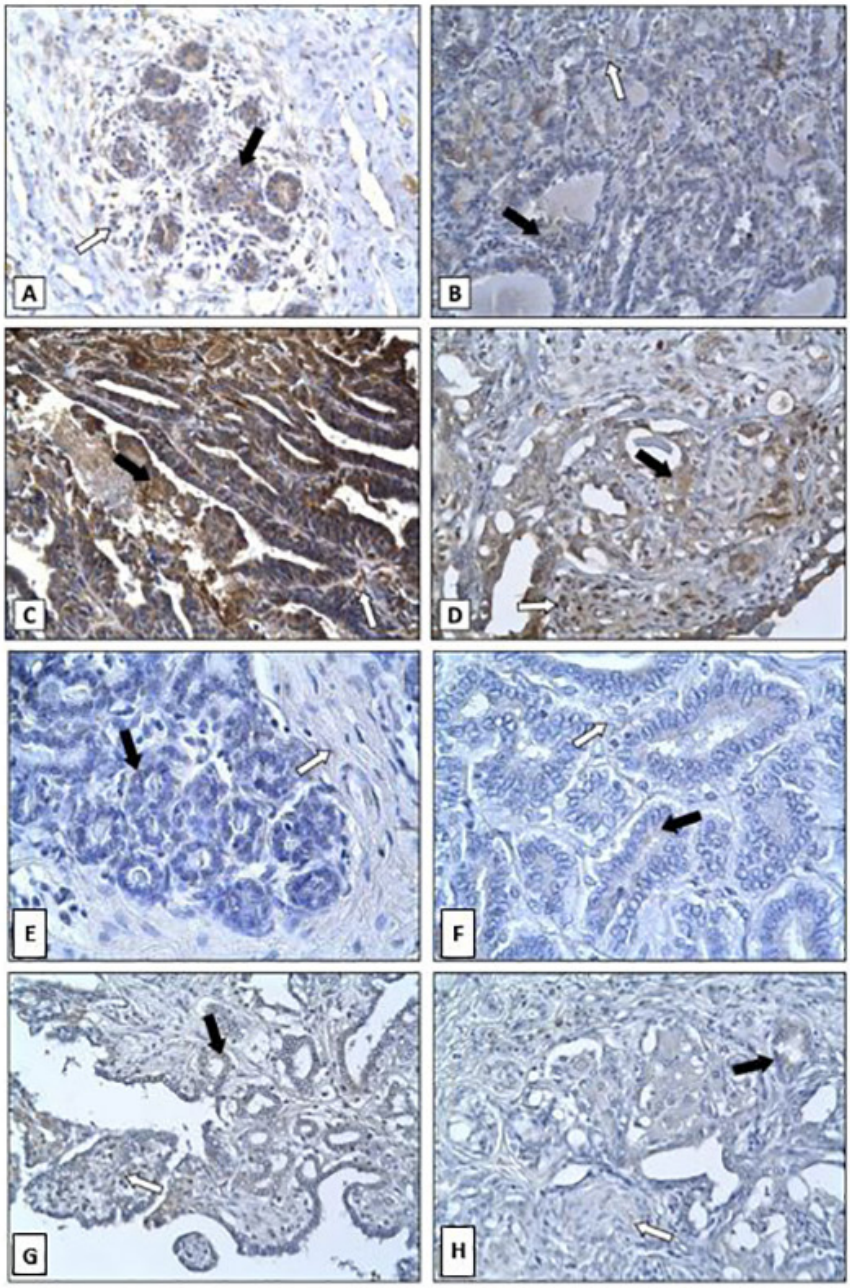

Figure 1. Photomicrographs of the canine mammary gland. Scores of the intensity of immunostaining for anti-MMP-9 (A–D) and anti-MMP-2 (E–H) in epithelial (full arrow) and stromal cells (empty arrow). A) Normal breast, 200x. Scores one and two in epithelial and stromal cells, respectively. B) Simple mammary adenoma, 200x. Score one in epithelial and stromal cells. C) Tubulopapillary carcinoma, 200x. Epithelial and stromal cells with score three. D) Complex carcinoma, 200x. Score two in epithelial cells and three in stromal cells. E) Normal breast, 200x. Rare epithelial and stromal cells with score one. F) Simple mammary adenoma, 400x. Score one in epithelial cells and absence of immunostaining in stromal cells. G) Tubulopapillary carcinoma, 100x. Epithelial and stromal cells with score one. H) Complex carcinoma, 200x. Score one in epithelial and stromal cells.

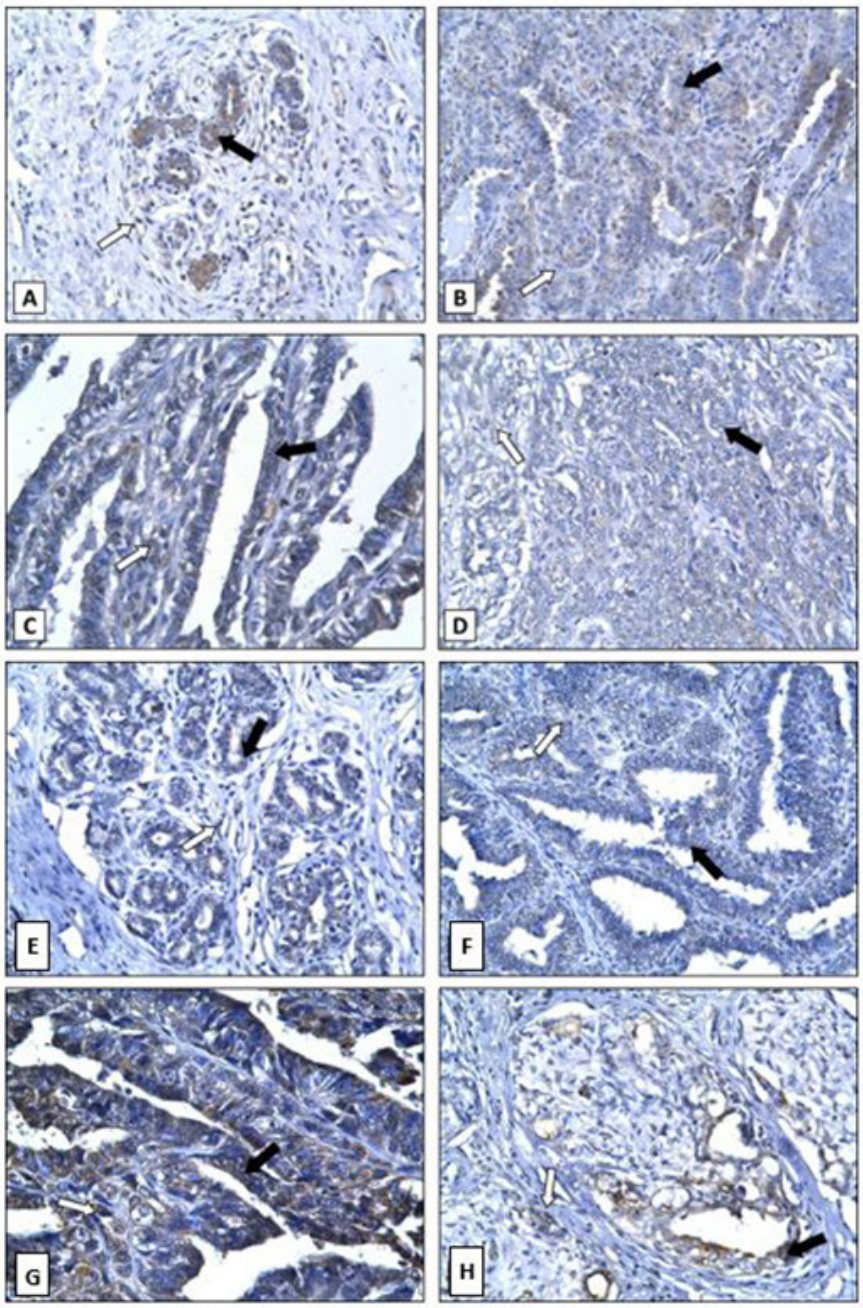

Figure 2. Photomicrographs of the canine mammary gland. Scores of the intensity of immunostaining for anti-TIMP-1 (A–D) and anti-TIMP-2 (E–H) in epithelial (full arrow) and stromal cells (empty arrow). A) Normal breast, 200x. Epithelial and stromal cells with score two. B) Simple mammary adenoma, 200x. Score one in epithelial and stromal cells. C) Tubulopapillary carcinoma, 400x. Epithelial and stromal cells with score one. D) Complex carcinoma, 200x. Score one in the epithelial and stromal cells. E) Normal breast, 200x. Epithelial and stromal cells with score one. F) Simple mammary adenoma, 100x. Epithelial and stromal cells with score one. G) Tubulopapillary carcinoma, 200x. Epithelial and stromal cells with score three. H) Complex carcinoma, 100x. Immunostaining with score two in epithelial cells and one in stromal cells.

Regarding the intensity of immunostaining of MMP-9 in acinar epithelial and periacinar stromal cells, a difference was observed between normal and neoplastic mammary tissues with complex and tubulopapillary carcinoma ($p<0.05$), with slight immunostaining in the normal breast and moderate or accentuated in carcinomas. In contrast, no difference was observed between the evaluated samples relative to the number of acinar epithelial and periacinar stromal cells labeled for MMP-9 ($p>0.05$), with a marked number of labeled cells in most samples. Moreover, no difference in the immunostaining of MMP-2 in acinar epithelial and periacinar stromal cells was found between normal and neoplastic canine mammary tissue samples, with the majority presenting a slight intensity of immunostaining and a slight number of labeled cells (Table 1).

The intensity of immunostaining of TIMP-1 was higher in periacinar stromal cells of normal mammary tissue ($p<0.05$) compared to complex carcinomas. Immunostaining of TIMP-1 was observed in the acinar epithelium, periacinar fibroblasts, and inflammatory cells (lymphocytes) in all samples. For TIMP-2, the intensity of immunostaining in acinar epithelial cells was high ($p<0.05$) in tubulopapillary carcinomas compared to the normal breast (Table 2).

A positive correlation was found between MMP-9 and TIMP-1 in normal mammary glands regarding the intensity of immunostaining in epithelial ($r=0.5552$; $p=0.0136$) and stromal cells ($r=0.4708$; $p=0.0362$), as well as for the number of immunostained epithelial ($r=0.4720$; $p=0.0356$) and stromal cells ($r=0.5259$; $p=0.0207$). A strong positive correlation was also observed between MMP-9 and TIMP-2 regarding the intensity of immunostaining in epithelial cells of the tubulopapillary carcinoma ($r=0.7531$; $p=0.0098$).

Table 1. Distribution of cases according to the scores applied to the intensity of immunostaining and number of cells immunostained with anti-MMP-9 and anti-MMP-2 antibodies in acinar epithelial and periacinar stromal cells relative to the diagnosis

MMP-9					
Intensity of immunostaining in acinar epithelial cells					
	Diagnosis	**Normal[A] % (n=24)**	**SMA[B] % (n=7)**	**TPC[C,A] % (n=17)**	**CC[D,A] % (n=12)**
Score	**Absent**	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
	Slight	75 (18)	14.3 (1)	17.6 (3)	16.7 (2)
	Moderate	16.6 (4)	71.4 (5)	47.1 (8)	50 (6)
	Accentuated	8.4 (2)	14.3 (1)	35.3 (6)	33.3 (4)
Intensity of immunostaining in periacinar stromal cells					
	Diagnosis	**A**	**B**	**C,A**	**D,A**
	Absent	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
Score	**Slight**	79.1 (19)	71.4 (5)	35.2 (6)	33.3 (4)
	Moderate	20.2 (5)	14.3 (1)	47.2 (8)	25 (3)
	Accentuated	0.0 (0)	14.3 (1)	17.6 (3)	41.7 (5)
Number of immunostained acinar epithelial cells					
	Diagnosis	**A**	**B**	**C**	**D**
	Absent	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
Score	**Slight**	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
	Moderate	12.5 (3)	43 (3)	6 (1)	16.7 (2)
	Accentuated	87.5 (21)	57 (4)	94 (16)	83.3 (10)
Number of immunostained periacinar stromal cells					
	Diagnosis	**A**	**B**	**C**	**D**
	Absent	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
Score	**Slight**	16.6(4)	28.5 (2)	17.6 (3)	8.3 (1)
	Moderate	41.7 (10)	57 (4)	41.2 (7)	25 (3)
	Accentuated	41.7 (10)	14.5 (1)	41.2 (7)	66.7 (8)
MMP-2					
Intensity of immunostaining in acinar epithelial cells					
	Diagnosis	**Normal[A] % (n=23)**	**SMA[B] % (n=6)**	**TPC[C] % (n=13)**	**CC[D] % (n=20)**
Score	**Absent**	8.6 (2)	50 (3)	15.4 (2)	20 (4)
	Slight	56.5 (13)	50 (3)	69.2 (9)	60 (12)
	Moderate	30.4 (7)	0.0 (0)	15.4 (2)	5 (1)
	Accentuated	4.3 (1)	0.0 (0)	0.0 (0)	15 (3)
Intensity of immunostaining in periacinar stromal cells					
	Diagnosis	**A**	**B**	**C**	**D**
	Absent	13 (3)	16.6 (1)	30.7 (4)	25 (5)
Score	**Slight**	47.9 (11)	83.4 (5)	69.3 (9)	55 (11)
	Moderate	34.8 (8)	0.0 (0)	0.0 (0)	20 (4)
	Accentuated	4.3 (1)	0.0 (0)	0.0 (0)	0.0 (0)
Number of immunostained acinar epithelial cells					
	Diagnosis	**A**	**B**	**C**	**D**
	Absent	8.7 (2)	16.6 (1)	15.4 (2)	20 (4)
Score	**Slight**	34.8 (8)	83.4 (5)	46.2 (6)	45 (9)
	Moderate	26 (6)	(0)	23 (3)	20 (4)
	Accentuated	30.5 (7)	(0)	15.4 (2)	15 (3)
Number of immunostained periacinar stromal cells					
	Diagnosis	**A**	**B**	**C**	**D**
	Absent	13 (3)	33.4 (2)	30.8 (4)	25 (5)
Score	**Slight**	43.4 (10)	66.6 (4)	38.4 (5)	45 (9)
	Moderate	21.8 (5)	0.0 (0)	23.1 (3)	25 (5)
	Accentuated	21.8 (5)	0.0 (0)	7.7 (1)	5 (1)

SMA-simple mammary adenoma; TPC-tubulopapillary carcinoma; CC-complex carcinoma. More than one letter in the Diagnosis row means statistical difference ($p<0.05$) between the letters the group represents.

Table 2. Distribution of cases according to the scores applied to the intensity of immunostaining and number of cells immunostained with anti-TIMP-1 and anti-TMP-2 antibodies in acinar epithelial and periacinar stromal cells relative to the diagnosis

		TIMP-1			
Intensity of immunostaining in acinar epithelial cells					
	Diagnosis	**Normal[A] % (n=21)**	**SMA[B] % (n=6)**	**TPC[C] % (n=17)**	**CC[D] % (n=16)**
Score	**Absent**	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
	Slight	71.4 (15)	83.3(5)	70.6 (12)	75 (12)
	Moderate	28.6 (6)	0.0 (0)	23.5 (4)	19 (3)
	Accentuated	0.0 (0)	16.7 (1)	5.9 (1)	6 (1)
Intensity of immunostaining in periacinar stromal cells					
	Diagnosis	**A**	**B**	**C**	**D,A**
Score	**Absent**	0.0 (0)	0.0 (0)	5.9 (1)	12.5 (2)
	Slight	66.6 (14)	100 (6)	70.6 (12)	87.5 (14)
	Moderate	33.4 (7)	0.0 (0)	23.5 (4)	0.0 (0)
	Accentuated	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
Number of immunostained acinar epithelial cells					
	Diagnosis	**A**	**B**	**C**	**D**
Score	**Absent**	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
	Slight	23.8 (5)	16.6 (1)	41 (7)	37.5 (6)
	Moderate	33.3 (7)	50 (3)	35.2 (6)	37.5 (6)
	Accentuated	42.8 (9)	33.4 (2)	23.8 (4)	25 (4)
Number of immunostained periacinar stromal cells					
	Diagnosis	**A**	**B**	**C**	**D**
Score	**Absent**	0.0 (0)	0.0 (0)	5.9 (1)	12.6 (2)
	Slight	47.6 (10)	50 (3)	70.6 (12)	56.2 (9)
	Moderate	23.8 (5)	50 (3)	23.5(4)	25 (4)
	Accentuated	28.6 (6)	0.0 (0)	0.0 (0)	6.2 (1)
		TIMP-2			
Intensity of immunostaining in acinar epithelial cells					
	Diagnosis	**Normal[A] % (n=20)**	**SMA[B] % (n=6)**	**TPA[C,A] % (n=18)**	**CC[D] % (n=14)**
Score	**Absent**	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
	Slight	95 (19)	83.3 (5)	66.7 (12)	57.2 (8)
	Moderate	5 (1)	16.7 (1)	22.2 (4)	35.7 (5)
	Accentuated	0.0 (0)	0.0 (0)	11.1 (2)	7.1 (1)
Intensity of immunostaining in periacinar stromal cells					
	Diagnosis	**A**	**B**	**C**	**D**
Score	**Absent**	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
	Slight	95 (19)	66.6 (4)	61.1 (11)	64.3 (9)
	Moderate	5 (1)	33.3 (2)	27.8 (5)	28.6 (4)
	Accentuated	0.0 (0)	0.0 (0)	11.1 (2)	7.1 (1)
Number of immunostained acinar epithelial cells					
	Diagnosis	**A**	**B**	**C**	**D**
Score	**Absent**	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
	Slight	25 (5)	16.7 (1)	16.7 (3)	21.4 (3)
	Moderate	60 (12)	66.6 (4)	50 (9)	42.9 (6)
	Accentuated	15 (3)	16.7 (1)	33.3 (6)	35.7 (5)
Number of immunostained periacinar stromal cells					
	Diagnosis	**A**	**B**	**C**	**D**
Score	**Absent**	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
	Slight	60 (12)	83.3 (5)	61.1 (11)	64.3 (9)
	Moderate	35 (7)	16.7 (1)	38.9 (7)	28.6 (4)
	Accentuated	5 (1)	0.0 (0)	0.0 (0)	7.1 (1)

SMA-simple mammary adenoma; TPC-tubulopapillary carcinoma; CC-complex carcinoma. More than one letter in the Diagnosis row means statistical difference ($p<0.05$) between the letters the group represents.

Discussion

Studies on tumor biological behavior have focused on stromal tissue, with its role in tumorigenesis, tumor invasion, and metastasis formation being considerable[29, 30]. MMP and TIMP play an important role in this mechanism, as they participate in the process of stromal degradation and components of the basement membrane, facilitating the dissemination of neoplastic cells[31].

Regarding MMP-9, a difference was observed between normal mammary tissue and malignant neoplastic tissue, suggesting the involvement of this enzyme in the development of canine mammary cancer. Similarly, Hirayama *et al*.[25] and Kawae *et al*.[24] observed high immunostaining of MMP-9 in carcinomas. Gramulia *et al*.[33] reported a diffuse or granular cytoplasmic pattern of MMP-9 in carcinomas, which was also observed in this research. In addition, Loukopoulos *et al*.[32] described marked immunostaining of this enzyme in carcinomas, which reiterates the importance of its increase in canine mammary tissue with malignant proliferation, which may be related to the process of tissue invasion by neoplastic cells. In this sense, MMP-9 is important in neoplastic development, but its immunostaining profile is variable.

No difference was observed between the groups in the evaluation of the number of epithelial and stromal cells labeled for MMP-9, which is in agreement with the findings of Pellikainen *et al*.[34] and Aresu *et al*.[26]. However, similar to the results observed in this research, these authors describe a certain degree of cytoplasmic immunostaining of MMP-9 in normal epithelial and stromal cells, as well as in neoplastic epithelial cells. Therefore, the intensity of immunostaining is considered as a valuable evaluation tool for MMP-9 in comparison to the number of labeled cells, especially when considering malignant neoplastic cells, as they commonly have genetic heterogeneity[35, 36] and clonal expansion capacity[37, 38], which allows the proliferation of cells with a high capacity for MMP-9 synthesis, allowing a high degradation of the basement membrane (BM) and ECM, and facilitating tumor invasion.

The MMP activity in canine mammary tumors is mainly associated with malignancy[26, 39]. Santos *et al*.[40] concluded that MMP-9 and Ki-67 are independent prognostic markers in malignant mammary tumors, suggesting that the high immunoexpression of MMP in the postoperative period in aggressive malignant tumors can be studied as a therapeutic target. Yokta *et al*.[41] concluded that concentrations of this MMP could be four to 26 times higher in canine mammary carcinomas than in normal mammary tissue. The results of this study corroborate this hypothesis, especially regarding the expression of MMP-9, which was more pronounced in epithelial and stromal cells of complex and tubulopapillary carcinomas than in the normal mammary gland.

The evaluation of the immunostaining of MMP-2 showed no difference between the evaluated samples regarding the intensity and number of immunostained epithelial and stromal cells. On the contrary, Aresu *et al*.[26] demonstrated high levels of pro-MMP-2 and MMP-2 by the zymography and immunohistochemistry methods, respectively, in carcinomas compared to mammary adenomas in bitches. However, the authors made

no distinction regarding the histomorphological type, evaluating together the different samples diagnosed with carcinoma. Thus, the absence of statistical difference in the present study could be attributed to the types of evaluated tumors, which may be less influenced by MMP-2 compared to MMP-9, which exhibited higher intensity of immunostaining in carcinomas.

According to Pellikainen *et al*.[34], the immunoexpression of MP-1 and TIMP-2 in tissues contributes to the activation and regulation of MMP-2 and can alter its participation in different neoplastic processes. Papparella *et al*.[39] describe the immunostaining of MMP-2 in normal and neoplastic epithelial cells, as well as in stromal cells, as observed in this study.

The low intensity of immunostaining of TIMP-1 was observed in periacinar stromal cells of complex carcinoma compared to the normal breast. On the other hand, Aresu *et al*.[26] described no difference in the immunostaining of TIMP-1 between normal and neoplastic canine mammary tissue. Kawai *et al*.[24] demonstrated high immunoexpression of this protein in carcinomas, followed by adenomas and normal breast. In humans, TIMP-1 is described as an inhibitor of tumor cell invasion and metastasis. As an example, Nakopoulou *et al*.[42] evaluated the immunohistochemical expression of TIMP-1 in 133 infiltrating breast carcinomas of women and observed increased immunostaining in cancer cells in 60.15% of the cases, which is inversely related to the histological degree, inversely proportional to cell proliferation, and its overexpression in neoplastic cells a determining factor for favorable prognosis in women. Thus, in this study, the reduction of the immunostaining of TIMP-1 and the absence of difference of TIMP-2 in complex carcinoma may indicate a high degradation of ECM and tumor progression in the referred histomorphological type.

High immunostaining was observed in the epithelial cells of the tubulopapillary carcinoma compared to those of the normal breast when evaluating the intensity of immunostaining of TIMP-2 relative to the diagnosis. The increased immunoexpression of TIMP is still controversial. TIMP-2 inhibits the activity of MMP and it is expected that high levels of this inhibitor preclude tumor progression, which, therefore, implies a better prognosis for patients with neoplasms[13]. However, evidence suggests that TIMP are multifunctional proteins that, in addition to their inhibitory effect on MMP, also promote the proliferation of some cell types, and their anti-apoptotic effects may favor the initial growth of the primary tumor[44].

In women, increased immunoexpression of TIMP-2 is also correlated with higher tumor aggressiveness and worse prognosis[16, 44]. In this context, high intensity of TIMP-2 in tubulopapillary carcinoma may be related both to the inhibition of the activity of MMP and to the promotion of tumor growth and ECM degradation. However, the proliferative action of TIMP-2, together with the proteolytic action of MMP-9, may overlap the inhibitory action of TIMP-2 and, thus, represent tumor evolution and invasion, as a positive correlation was observed between these proteins. Also, significant immunostaining restricted to tubulopapillary carcinoma suggests the relationship between TIMP-2 and this histomorphological type, as it occurs in humans, where the increase in this protein has been associated with the ductal histological type[45, 46].

Therefore, the results show that the balance between metalloproteinases and their inhibitors in the normal canine breast is an essential condition for maintaining homeostasis, as only a positive correlation between MMP-9 and TIMP-1 was observed in normal mammary tissue. Thus, in light of these results, the increased immunostaining of TIMP and MMP, especially MMP-9 and TIMP-2, may compose an important factor in tumor evolution and the occurrence of tissue invasion relative to tubulopapillary and complex carcinomas in bitches.

Conclusion

Balanced activity between MMP-9, MMP-2, TIMP-1, and TIMP-2 maintains normal canine mammary tissue homeostasis, while an increased immunoexpression of MMP-9 and TIMP-2 and reduced immunoexpression of TIMP-1 in carcinomas suggest a favorable condition for tumor evolution and invasion. Also, tissue concentration of MMP and its inhibitors in the breast of bitches vary according to the tumor type, the degree of malignancy, and the adjacent neoplastic and stromal cells, which may reveal a little more about the behavior of each neoplastic type and assist future studies regarding diagnosis, treatment, and prognosis.

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