



Is the expression of matrix metalloproteinases (MMP-2, -9) and tissue inhibitors of metalloproteinases (TIMP-1, -2, and -3) associated with angiogenesis and clinicopathological features for breast cancer?

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ABSTRACT

Introduction: Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) are involved in the progression of several tumors, including breast cancer. Our aim was to investigate the association of immunohistochemical expression of protein MMP-2, and -9 and tissue inhibitors TIMP-1, -2, -3 by tumoral cells in the process of angiogenesis and to define their relation with clinicopathological features for breast cancer.

Methods: Immunohistochemical analysis of MMP-2, -9, TIMP-1, -2, -3, endoglin/CD105, estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) status was performed on 79 tissue samples of breast cancer with axillary lymph node dissection.

Results: Statistically significant difference was found between mean age of patients and tissue inhibitors of metalloproteinase (TIMP-1) expression status ($p=0.008$), i.e., women with TIMP-1 negative tumors were on average younger (mean age 46.5) compared to women with TIMP-1 positive tumors (mean age 58.1); TIMP-2 expression status showed association with ER status ($p=0.017$), while TIMP-3 negative tumors were on average more frequently ER and PR negative ($p=0.016$; $p=0.027$). Status of protein expression of MMP-9 was associated with TIMP-1 protein expression status ($p=0.033$), i.e., breast cancers with overexpression of protein MMP-9 were more frequently TIMP-1 protein positive.

Conclusion: Only TIMPs were associated with clinicopathological features for breast cancer. TIMP-2 expression was associated with worse (TIMP-2 positive tumors were frequently ER-negative), while TIMP-3

expression in tumoral cells was associated with better clinicopathological features for breast cancer (TIMP-3 positive tumors were frequently ER- and PR-positive).

Keywords: Breast cancer; matrix metalloproteinases; tissue inhibitors of metalloproteinases; angiogenesis; endoglin; immunohistochemistry

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INTRODUCTION

Considering the fact that extracellular matrix (ECM) plays an important role in cancer progression and that matrix metalloproteinases (MMPs) are main molecules responsible for its remodeling, over the past years many studies have observed their influence to cancer progression. Since their discovery, there have been attempts to develop MMP inhibitor programs, especially for breast cancer, which as a multifactorial and heterogeneous disease is urging for more targeted and personalized therapy (1). MMPs are main regulators during several phases of the angiogenic process, from deposition and remodeling of ECM components to cell proliferation and migration (2). They contribute to angiogenesis either by degrading basement membranes or by promoting and maintaining the angiogenic phenotype (1,3). In breast cancer progression, particular importance was given to MMP-2 and MMP-9 due to their specificity for Collagen IV and possible implication in metastatic spread (4,5).

Although the results of a large number of studies showed a correlation between high levels of MMPs with a more aggressive form of disease and shorter overall survival period (4,6,7), some studies reported conflicting results (8,9).

The results are even more inconsistent when it is about the main tissue inhibitors of MMPs. TIMPs, as well as MMPs, are secreted by tumoral and stromal cells. Increased TIMP expression may also mean a stromal response to a tumor invasion, or may indicate a tissue response during the control of the activity of the MMP in maintaining the integrity of the ECM. TIMPs have an anti-MMP activity which are responsible for tumor suppression but can also stimulate growth or influence the apoptosis. The balance between anti-MMP and antiapoptotic effect on tumor growth may depend on the amount of bioavailable TIMP proteins in the tumor microcirculation. Due to their main biological role, it is expected that increased levels of TIMPs inhibit tumor invasion and formation of distant metastases and thus improve prognosis (10). However, elevated TIMP levels are reported in association with cancer progression and identified as poor prognostic indicators in several human tumor types, including breast cancer (11,12). Recent studies have found TIMP-1 serum

levels enhanced in patients with triple-negative breast cancer (TNBC) and associated with poor prognosis (12), while others suggest TIMP-2 as a novel biological therapy for TNBC (13).

Having in mind all those facts, we aimed to investigate the possible association of immunohistochemical expression of protein MMP-2, MMP-9, TIMP-1, -2, and -3 by breast carcinoma cells with angiogenesis and to compare it with standard clinicopathological features for breast cancer in Bosnian women.

METHODS

Clinicopathological data

The biopsy samples of 79 patients with invasive breast cancer (IBC) were diagnosed at the Department of Pathology, School of Medicine, Sarajevo, Bosnia and Herzegovina. All patients with IBC underwent partial or total mastectomy with axillary lymph node dissection. Patients who received neoadjuvant chemotherapy or radiotherapy, or had distant metastases at the time of diagnosis were excluded from the study.

Mean age of the patients at the time of diagnosis was 56.08 (range from 30 to 87).

All clinicopathological data are summarized in Table 1.

Immunohistochemical procedures

Protein expression of MMP-2, MMP-9, TIMP-1, TIMP-2, TIMP-3, Endoglin (CD105), estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) was determined by immunohistochemistry. All the tissue specimens of the breast with IBC were fixed in 10% neutral formalin and embedded in paraffin.

Briefly, four-micron-thick paraffin sections were mounted on poly-D-lysine coated slides and heated overnight at 60°C. The sections were deparaffinized in xylene, rehydrated in a decreasing series of ethanol solutions (100%, 90%, and 80%) for 5 min each and washed 2 times in 0.05 mol/L phosphate-buffered saline (PBS) (pH 7.4). To enhance antigen retrieval the sections were pretreated in a water bath with Trisethylenediaminetetraacetic acid (EDTA) buffer (pH 9.0) for 15 min at 95–97°C except for primary antibodies anti-CD105 and antiprogesterone

TABLE 1. Clinicopathological characteristics of 79 studied cases with primary breast carcinoma

Variables	n (%)
Age groups	
<50	19 (24.05)
≥50	60 (75.95)
Histological type	
Ductal	71 (89.87)
Lobular	8 (10.13)
Tumor grade (G)	
G1	15 (18.99)
G2	38 (48.10)
G3	26 (32.91)
Tumor size (pT)	
pT1	26 (32.91)
pT2	41 (51.90)
pT3	9 (11.39)
pT4	3 (3.80)
Nodal status	
Negative	35 (44.30)
Positive	44 (55.70)
Lymphovascular invasion (Lvi)	
Absent	37 (46.84)
Present	42 (53.16)
ER status	
Negative	29 (36.71)
Positive	50 (63.29)
PR status	
Negative	33 (41.77)
Positive	46 (58.23)
HER2 expression	
Negative	58 (73.42)
Positive	21 (26.58)
Level of HER2 expression	
0	43 (54.43)
1+	15 (18.99)
2+	8 (10.13)
3+	13 (16.45)
MMP-2 expression	
Negative	28 (35.44)
Positive	51 (64.56)
MMP-9 expression	
Negative	6 (7.59)
Positive	73 (92.41)
TIMP-1 expression	
Negative	20 (25.32)
Positive	59 (74.68)

(Contd...)

TABLE 1. (Continued)

Variables	n (%)
TIMP-2 expression	
Negative	63 (79.75)
Positive	16 (20.25)
TIMP-3 expression	
Negative	4 (5.06)
Positive	75 (94.94)

ER: Estrogen receptor, PR: Progesterone receptor, HER2: Human epidermal growth factor receptor-2, MMP-2: Matrix metalloproteinase-2, MMP-9: Matrix metalloproteinase-9; TIMP-1: Tissue inhibitor of metalloproteinase-1, TIMP-2: Tissue inhibitor of metalloproteinase-2, TIMP-3: Tissue inhibitor of metalloproteinase-3

receptor which slides were pretreated in the microwave oven in an EDTA buffer solution (pH 9.0) 3 times for 5 min each (1000 W/s). Endogenous peroxidase activity was blocked by incubating the sections in 0.3% hydrogen peroxide (H₂O₂) in methanol for 10 min, and to reduce the nonspecific binding capacity of the tissue; slides were then washed in PBS 2 times for 5 min at room temperature. Afterward, the sections were incubated with one of the respective primary antibodies obtained from Leica Biosystems, Newcastle Ltd.: As mouse monoclonal: Anti-MMP2 (NCL-MMP2-507, clone 17B11, dilution 1:50), anti-TIMP2 (NCL-TIMP2-487, clone 46E5, dilution 1:20), anti-TIMP3 (NCLTIMP3, clone 18D12b, dilution 1:30), and anti-CD105 (NCL-CD105, clone 4G11, dilution 1:50), and from DakoCytomation, Glostrup, Denmark: Polyclonal rabbit antihuman MMP9 (code A0150, dilution 1:50), as well as monoclonal mouse: Anti-human TIMP1 (code M7293, clone VT7, dilution 1:50), anti-human ER- α (code M7047, clone 1D5, dilution 1:30), anti-human PgR (clone PgR 636, dilution 1:30), and also HercepTest™ (code K5204). The sections were washed 3 times in PBS (2 min each). As staining detection system it was used CSA II/HRP mouse (code K1497; Dako, Glostrup, Denmark) for MMP-2 and TIMP-3, CSA I rabbit (code K1501) and CSA II/HRP mouse (code K1497; Dako, Glostrup, Denmark) for MMP-9, as well as EnVision Detection Kit (Dako, Glostrup, Denmark) for TIMP-1, TIMP-2, and CD105 for 30 min. The slides were washed in PBS 3 times for 2 min, incubated with 3,3'-diaminobenzidine solution (DAB;

Dako, Cytomation Inc.) as chromogen for 10 min at ambient temperature until the color reaction was revealed, then washed 3 times in distilled water (5 min each). Finally, the sections were counter-stained with Mayer's hematoxylin for 2 min, washed thoroughly in running tap water, dehydrated with ethanol, clarified in xylol and mounted with Eukitt.

Immunohistochemical evaluation

The evaluation of immunohistochemical staining was carried out blind to the patient's data and pathological features.

Estimation of protein expression of MMP, TIMP, ER, PR, and HER-2 in breast carcinoma cells, as well as estimation of CD105 positive newly formed blood vessels in tumor tissue, was performed by the semi-quantitative method.

The staining intensity of protein MMP-2, MMP-9, TIMP-1, TIMP-2, and TIMP-3 and the number of stained tumor cells were both taken into consideration. Stains were scored on a scale of 0-3: Score 0, was assigned for no staining; Score 1, if cytoplasm and cell membrane weakly stained in <10% tumor cells; Score 2, for weak to moderate staining in 10–30% of tumor cells; and Score 3, if more than 30% of tumor cells strongly stained. The immunohistochemical expression was classified as positive or negative, considering 0 and 1 as negative and 2 or 3 as positive. Samples were considered to be MMP-2, MMP-9, TIMP-1, TIMP-2, and TIMP-3 positive when $\geq 10\%$ of tumor cells were immunoreactive.

ER and PR positivity were defined as any positive nuclear staining (i.e., $\geq 1\%$) (14).

HER-2 immunolabeling was measured according to the HercepTest scoring system (DakoCytomation) as follows: 0- no staining or faint incomplete membranous staining in <10% cells; 1- faint incomplete membranous staining in >10% cells, 2- weak to moderate complete staining in >10% cells, and 3- strong complete staining in >10% cells. Cases scored as 2+ were considered equivocal, and retested using chromogen *in situ* hybridization.

Quantification of angiogenesis

Sections stained with anti-CD105 antibody were used for the quantification of newly formed blood vessels

in breast carcinoma tissue. The microscopic fields of breast carcinoma were observed by magnification $\times 200$. Newly formed, CD105 positive blood vessels with visible vascular lumen were counted in 10 visual fields, separately. The total number of newly formed blood vessels was quantified as the mean value for each tumor sample and expressed as the total number of newly formed blood vessels by the high power field magnification. Isolated cells or vascular cells without visible lumen were not taken into account.

Statistical analysis

Statistical analysis was performed using SPSS version 21.0 (SPSS Inc., Chicago, IL).

Normality of the distribution of numerical variables was tested using Kolmogorov–Smirnov, or Shapiro–Wilk test, where appropriate. Accordingly, all variables are presented by the appropriate measures of central tendency - arithmetic mean (\pm standard deviation) or median (with interquartile range). The values of categorical variables are presented in absolute numbers. T-test, Mann–Whitney U-test, and ANOVA (with Bonferroni multiple comparisons) were used to test the statistical significance in differences in respective central tendency measures of the numerical variables between sub-groups (according to clinicopathological features for breast cancer and the status of protein MMP-2, MMP-9, TIMP-1, TIMP-2, and TIMP-3). Chi-square test (or Fisher's exact test, where appropriate) was used to test the dependence between individual categorical variables (i.e., clinicopathological features for breast cancer and the status of the tissue expression of MMP-2, MMP-9, TIMP-1, TIMP-2, and TIMP3). $p = 0.05$ or less was considered statistically significant for all statistical analyses.

RESULTS

Protein expression of MMP-2, MMP-9, TIMP-1, TIMP-2, and TIMP-3 in breast cancer tissue

MMP-2, MMP-9, TIMP-1, TIMP-2, and TIMP-3 proteins were mainly expressed in the cytoplasm or membranes of tumor cells, as well as protein CD105/Endoglin in endothelial cells of newly formed blood vessels of breast cancer tissue (Figure 1).

The expression of MMPs (MMP-2 and MMP-9), tissue inhibitors of MMPs (TIMP-1, TIMP-2, and

TIMP-3) in cancer cells, and the mean number of CD105 newly formed blood vessels was observed in relation to the distribution of clinicopathological features for breast cancer.

Relationship between MMP protein expression in breast cancer tissue and clinicopathological features

Protein expression of MMP-2 and MMP-9 in breast cancer cells showed no relation to patients age, tumor size, tumor grade, lymphovascular invasion, lymph node status, nor to estrogen receptor (ER), progesteron receptor (PR) status and HER2 status (Table 2).

Relationship between TIMP protein expression in breast cancer tissue and clinicopathological features

Statistically significant difference in mean age of patients was found to exist with respect to TIMP-1 expression status ($p = 0.008$), i.e., women

with TIMP-1 negative expression were on average younger (mean age 46.5) compared to women with TIMP-1 positive expression (mean age 58.1). TIMP-1 expression status showed association with patients' age group (<50 or ≥ 50 years), i.e. patients aged ≥ 50 were on average more frequently TIMP-1 positive ($p = 0.000$), while to other clinicopathological features showed no relation.

TIMP-2 expression status was associated with ER status ($p = 0.017$), i.e., TIMP-2 positive tumors were on average more frequently ER-negative, while TIMP-2 negative tumors were more frequently ER-positive (Table 2).

TIMP-3 expression status showed association with estrogen and progesterone receptor status; TIMP-3 negative tumors were on average more frequently ER- and PR-negative ($p = 0.016$ and $p = 0.027$, respectively) (Table 2).

Relationship between mean number of CD105 newly formed blood vessels in breast cancer tissue and clinicopathological features

Statistically significant difference in a mean number of CD105 newly formed blood vessels, and tumor size (pT) was found to exist (between pT1 and pT2, $p = 0.017$) (Table 2).

Regarding the tumor grade (G), statistically significant difference in a mean number of CD105 newly formed blood vessels were shown to exist between tumors of G1 and G2 ($p = 0.002$) and between tumors of G1 and G3 ($p = 0.002$) (Table 2).

The mean number of newly formed blood vessels was higher in ER-negative and PR-negative tumors (compared to ER- and PR-positive tumors), ($p = 0.002$, and $p = 0.006$, respectively).

Relationship between MMP, TIMP protein expression, and CD105 newly formed blood vessels

An analysis of MMPs and TIMPs tissue expression revealed a significant association only between MMP-9 and TIMP-1. As shown in Table 3, the status of tissue expression of MMP-9 was associated with TIMP-1 tissue expression status ($p = 0.033$). We found that breast cancers with overexpression of protein MMP-9 were more frequently TIMP-1 protein positive. However, no statistically significant

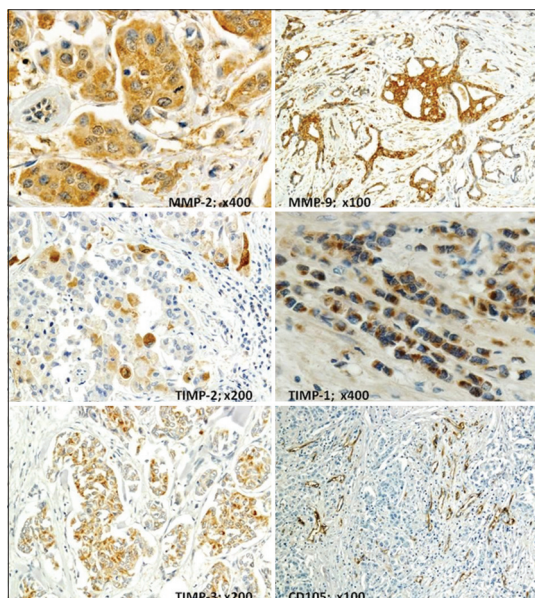


FIGURE 1. Immunohistochemical staining of proteins matrix metalloproteinases (MMP-2), MMP-9, tissue inhibitors of metalloproteinases (TIMP-1), TIMP-2, and TIMP-3 in the cytoplasm and membranes of breast cancer cells. CD105/Endoglin positive newly formed blood vessels in the IBC tissue, lined by a single layer of immunopositive flattened endothelial cells with a visible vascular lumen.

TABLE 2. Distribution of clinicopathological parameters for breast cancer in relation to the status of the tissue expression of MMP-2, MMP-9, TIMP-1, TIMP-2, TIMP-3 and the number of CD105 positive newly formed blood vessels

Clinicopathological parameters/ prognostic predictive factors	MMP 2		MMP9		TIMP1		TIMP2		TIMP3		CD105
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative	
Age (x±SD)	55.5±11.9	57.3±12.2	56.1±11.9	56.2±13.5	58.1±11.1	46.5±15.0 P=0.008	56.9±9.7	55.9±12.5	56.4±12.0	54.5±15.0	N/A
Age group											
<50	13	6	17	2	8	11	2	17	18	1	12.3±4.9
≥50	8	22	56	4	51	9 P=0.000	14	46	57	3	13.3 (10.3)
Tumor size											
pT1	16	10	25	1	22	4	6	20	24	2	9.5±5.6
pT2	26	15	38	3	29	12	8	33	41	0	13.5 (10.0)
pT3	6	3	8	1	6	3	1	8	7	2	11.3 (10.4)
pT4	3	0	2	1	2	1	1	2	3	0	19.8±3.9 P=0.017
Histological grade											
G1	8	7	15	0	12	3	2	13	14	1	6.4±3.6
G2	26	12	35	3	31	7	8	30	37	1	14.2 (9.7)
G3	17	9	23	3	16	10	6	20	24	2	13.4 (9.0) P=0.002
Lymphovascular invasion											
Present	31	11	37	5	31	11	9	33	40	2	13.0 (9.4)
Absent	20	17	36	1	28	9	7	30	35	2	12.7 (11.0)
Lymph node status											
Positive	28	16	40	4	31	13	10	34	43	1	13.1 (9.2)
Negative	23	12	33	2	28	7	6	29	32	3	10.0 (11.9)

(Contd...)

TABLE 2. (Continued)

Clinicopathological parameters/prognostic predictive factors	MMP 2		MMP9		TIMP1		TIMP2		TIMP3		CD105
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative	
Status of estrogen receptors											
Positive	30	20	46	4	40	10	6	44	50	0	10.9±5.9
Negative	21	8	27	2	19	10	10	19	25	4	16.2 (9.4)
								P=0.017		P=0.016	P=0.002
Status of progesterone receptors											
Positive	32	14	43	3	36	10	7	9	46	0	11.1±5.8
Negative	19	14	30	3	23	10	39	24	29	4	14.1 (13.1)
										P=0.027	P=0.006
HER2 expression											
0	26	17	13	0	32	11	4	39	41	2	13.2 (9.4)
1+	10	5	7	1	12	3	5	10	15	0	11.3±6.9
2+	4	4	12	3	6	2	2	6	8	0	15.8±8.3
3+	11	2	41	2	9	4	5	8	11	2	13.0 (8.8)
Status of HER2 expression											
Positive	15	6	20	1	15	6	7	14	19	2	13.1 (12.3)
Negative	36	22	53	5	44	14	9	49	56	2	12.7±7.8

MMP-2: Matrix metalloproteinase-2, MMP-9: Matrix metalloproteinase-9, TIMP-1: Tissue inhibitor of metalloproteinase-1, TIMP-2: Tissue inhibitor of metalloproteinase-2, TIMP-3: Tissue inhibitor of metalloproteinase-3, HER-2: Human epidermal growth factor receptor-2, N/A: Not applicable

TABLE 3. Distribution of matrix metalloproteinases, tissue inhibitors of metalloproteinases, and number of CD105 newly formed blood vessels

Variables	MMP-2		MMP-9		TIMP-1		TIMP-2		TIMP-3		CD105
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative	
MMP-2											
Positive	N/A	N/A	46	5	39	12	10	41	47	4	13.0 (9.3)
Negative	N/A	N/A	27	1	20	8	6	22	28	0	12.3 (11.7)
MMP-9											
Positive	46	27	N/A	N/A	57	16	16	57	49	4	13.0 (9.3)
Negative	5	1	N/A	N/A	2	4	0	6	6	0	11.4±7.6
						P=0.033					
TIMP-1											
Positive	39	20	57	2	N/A	N/A	12	47	55	4	13.0 (9.2)
Negative	12	8	16	4	N/A	N/A	4	16	20	0	12.9±7.5
				P=0.033							
TIMP-2											
Positive	10	6	16	0	12	4	N/A	N/A	15	1	13.3 (12.9)
Negative	41	22	57	6	47	16	N/A	N/A	60	3	17.4±12.8
TIMP-3											
Positive	47	28	69	6	55	20	15	60	N/A	N/A	12.8 (9.4)
Negative	4	0	4	0	4	0	1	3	N/A	N/A	12.6±3.9

MMP-2: Matrix metalloproteinase-2, MMP-9: Matrix metalloproteinase-9, TIMP-1: Tissue inhibitor of metalloproteinase-1, TIMP-2: Tissue inhibitor of metalloproteinase-2, TIMP-3: Tissue inhibitor of metalloproteinase-3, N/A: Not applicable

difference in a mean number of CD105 newly formed blood vessels was found to exist between different groups with respect to MMPs and TIMPs expression status.

DISCUSSION

The development of metastases is a complex process involving angiogenesis and degradation of ECM, which are essential for the spread and proliferation of cancer cells. These events include the activity of MMPs and their tissue inhibitors (TIMPs) which seem to be involved in the propagation of various tumors, including breast cancer (3,15). The expression of MMPs is generally very low and mainly regulated by transcription. Other mechanisms of their regulation include post-translational modification, latent-zymogen forms of MMPs, and coexpression of TIMPs. Many members of MMP family are dysregulated in human cancers, especially MMP-1, -2, 7, -9, and 13 (1). In a variety of studies related to MMPs and TIMPs in breast cancer, different methods of assessment were used, including immunohistochemistry and gene expression, its activity in

serum or plasma, or expression by mRNA *in situ* hybridization with the sole purpose to target them as diagnostic and therapeutic molecules in breast cancer (16).

In this study, we evaluated the significance of immunohistochemical expression of protein MMP-2, and -9 and their tissue inhibitors TIMP-1,-2, and -3 by tumoral cells in the process of angiogenesis, by comparing it with well-established clinicopathological features for breast carcinoma. The advantages of immunohistochemical evaluation are the direct readout of protein levels and the possibility to distinguish the expression of examined proteins in tumor versus stromal cells (16).

Immunohistochemical expression of the MMP-2 protein was positive in 64.56% of cancer cells, and MMP-9 protein in 92.41%. MMP-2 and MMP-9 status showed no association with clinicopathological features for breast cancer (Table 2).

Using immunohistochemistry, Vizoso et al. (17), Li et al. (18), and Talvensaari-Mattila et al. (19) reported opposite results in their studies, i.e., that the expression of metalloproteinases is related to

poor prognosis in breast cancer patients. Pellikainen et al. in the study of 421 patients found high MMP-9 expression in stromal cells to be prognostic for poor recurrence-free survival and breast cancer specific survival, while MMP-9 expression in tumoral cells was associated with smaller tumors and better survival outcomes in this cohort (20). Scorilas and colleagues found that lower expression of MMP-9 by tumoral cells is associated with poor prognostic factors, i.e., with tumors of greater diameter (8).

No statistically significant difference in the mean number of CD105 newly formed blood vessels was found to exist between different groups with respect to MMP-2 and MMP-9 (Table 3), although the higher number of CD105 positive newly formed blood vessels was recorded in MMP-2 and MMP-9 positive tumors ($p > 0.05$).

In general, TIMPs are known for their ability to inhibit MMP activity, but recently many MMP-independent functions of TIMPs have been discovered, thus highlighting their dual role in cell-ECM interactions and tumor behavior (21).

TIMPs are able to inhibit all active MMPs, but not with the same efficacy. TIMP-1 mostly inhibits MMP-7, MMP-9, MMP-1, and MMP-3, while TIMP-2 is the most effective inhibitor of MMP-2. TIMP-3 can inhibit MMP-2 and MMP-9 (22). In our study, the status of tissue expression of MMP-9 was associated with TIMP-1 tissue expression status ($p = 0.033$) (Table 3), which was expected due to previously mentioned facts. Jinga et al. pointed to the possibility that imbalance between MMP-9 and TIMP1 can be involved in the development of invasive breast carcinoma (4). By contrast, Thorsen et al., in a large study of 465 breast cancer patients examining the plasma concentration of MMP-9/TIMP-1 complex, found no correlation with disease-free survival (23). High serum and tumor levels of TIMP-1 have been reported in association with poor response to chemotherapy and decreased survival (24,25). Our results showed the statistically significant difference in mean age of patients with respect to TIMP1 expression status (i.e., between groups with positive and negative expression of TIMP-1) ($p = 0.008$). Women with TIMP-1 negative expression were on average younger (mean age 46.5) compared to women with TIMP-1 positive expression (mean

age 58.1). TIMP-1 expression status showed association with patients' age group (<50 or ≥ 50 years), i.e., patients aged ≥ 50 were on average more frequently TIMP-1 positive ($p = 0.000$) (Table 2).

TIMP-2 expression status showed association with ER status ($p = 0.017$), i.e., TIMP-2 positive tumors were on average more frequently ER-negative, while TIMP-2 negative tumors were more frequently ER-positive (Table 2). Same results showed other studies (11,26) once again indicating to the relation between TIMP-2 and aggressive behavior of breast cancer.

Among all TIMPs, TIMP-3 has the broadest spectrum of inhibition, and unlike others, TIMP-3 is tightly bounded to ECM (27). TIMP-3 expression status was associated with estrogen and progesterone receptor status; TIMP-3 positive tumors were on average more frequently ER- and PR-positive ($p = 0.016$ and $p = 0.027$, respectively) (Table 2). Measuring mRNA levels of TIMPs, Span et al. reported TIMP-3 as an only possible predictor for relapse-free survival in breast cancer patients, i.e., breast cancers with high TIMP-3 expression showed a better response to endocrine therapy (28). Likewise, Vizoso et al. have reported significantly higher TIMP-3 expression in ER-positive tumors. They also found that TIMP-3 expression by fibroblastic cells, but not by tumoral cells correlates positively with distant metastases (17).

We also investigated possible differences in angiogenesis, i.e., the mean number of CD105 positive newly formed blood vessels with regard to standard clinicopathological features for breast carcinoma. Statistically, significant difference was observed in a mean number of CD105 newly formed blood vessels between tumors of G1 and G2 ($p = 0.002$), and between tumors of G1 and G3 ($p = 0.002$) (Table 2), i.e., tumors of Grade 2 had on average more CD105 positive newly formed blood vessels compared to Grade 1 tumors as well as tumors of Grade 3 compared to Grade 1 tumors. An increase in the number of newly formed blood vessels was observed with increasing of tumor size, although statistically significant difference was found to exist only between tumors of pT1 and pT2 ($p = 0.017$) (Table 2), showing that tumors larger than 2 cm need more blood supply for progression. The mean number of newly formed blood vessels was higher

in ER-negative and PR-negative tumors (compared to ER- and PR-positive tumors), ($p = 0.002$, and $p = 0.006$, respectively), which confirmed the findings of previous studies and also indicate that ER- and PR-negative tumors are associated with more aggressive clinical behavior, higher histological grade and negative clinicopathological features for breast carcinoma (29).

CONCLUSIONS

To the best of our knowledge, this is the first study involving MMPs and TIMPs in breast cancer of Bosnian women. When it comes to tissue inhibitors of MMPs our results especially highlight the complex role of TIMPs in cancer progression as well as the complexity of TIMP-MMP interaction. In support of that are findings that some of TIMPs were associated with worse (i.e., TIMP-2) and some with a better pathological prognostic-predictive factor for breast carcinoma (i.e., TIMP-3), as well as the existence of MMP-TIMP dependency (i.e., MMP-9 and TIMP-1). Results of our study do not indicate a significant association of MMPs with angiogenesis (mean number of CD105 newly formed blood vessels) nor with clinicopathological features for breast cancer. Possible reasons for that could be relatively small sample size, ethnic differences, the usage of different antibodies or laboratory procedures compared to previous studies and the fact that we assessed the protein expression only in tumoral cells. Further studies, especially prospective ones, with greater sample size and the usage of different methods to assess MMPs and TIMPs in breast cancer, will be necessary to determine the impact of MMPs and TIMPs to progression and outcome in Bosnian women.

REFERENCES

- Cathcart J, Pulkoski-Gross A, Cao J. Targeting matrix metalloproteinases in cancer: Bringing new life to old ideas. *Genes Dis* 2015;2(1):26-34. <https://doi.org/10.1016/j.gendis.2014.12.002>.
- Yoon SO, Park SJ, Yun CH, Chung AS. Roles of matrix metalloproteinases in tumor metastasis and angiogenesis. *Biochem Mol Biol* 2003;36(1):128-37. <https://doi.org/10.5483/BMBRep.2003.36.1.128>.
- Holanda AO, Oliveira AR, Cruz KJ, Severo JS, Morais JB, Silva BB, et al. Zinc and metalloproteinases 2 and 9: What is their relation with breast cancer? *Rev Assoc Med Bras* (1992) 2017;63(1):78-84.
- Jinga DC, Blidaru A, Coundrea I, Ardeleanu C, Dragomir C, Szegli G, et al. MMP-9 and MMP-2 gelatinases and TIMP-1 and TIMP-2 inhibitors

in breast cancer: Correlations with prognostic factors. *J Cell Mol Med* 2006;10(2):499-510. <https://doi.org/10.1111/j.1582-4934.2006.tb00415.x>.

- Daniele A, Zito AF, Giannelli G, Divella R, Asselti M, Mazzocca A, et al. Expression of metalloproteinases MMP-2 and MMP-9 in sentinel lymph node and serum of patients with metastatic and non-metastatic breast cancer. *Anticancer Res* 2010;30(9):3521-7.
- McGowan PM, Duffy MJ. Matrix metalloproteinase expression and outcome in patients with breast cancer: Analysis of a published database. *Ann Oncol* 2008;19(9):1566-72. <https://doi.org/10.1093/annonc/mdn180>.
- Sivula A, Talvensaari-Mattila A, Lundin J, Joensuu H, Haglund C, Ristimäki A, et al. Association of cyclooxygenase-2 and matrix metalloproteinase-2 expression in human breast cancer. *Breast Cancer Res Treat* 2005;89:215-20. <https://doi.org/10.1007/s10549-004-0714-4>.
- Scorilas A, Karameris A, Arniogianni N, Ardavanis A, Bassilopoulos P, Trangas T, et al. Overexpression of matrix-metalloproteinase-9 in human breast cancer: A potential favourable indicator in node-negative patients. *Br J Cancer* 2001;84(3):1488-96. <https://doi.org/10.1054/bjoc.2001.1810>.
- Kulić A, Dedić Pavetić N, Vrbanc J, Sirotković-Skerlev M. Low serum MMP1 in breast cancer: A negative prognostic factor? *Biomarkers* 2012;17(5):416-21. <https://doi.org/10.3109/1354750X.2012.678885>.
- Jiang Y, Goldberg ID, Shi YE. Complex roles of tissue inhibitors of metalloproteinases in cancer. *Oncogene* 2002;21(14):2245-52. <https://doi.org/10.1038/sj.onc.1205291>.
- Remacle A, McCarthy K, Noël A, Maguire T, McDermott E, O'Higgins N, et al. High levels of TIMP-2 correlate with adverse prognosis in breast cancer. *Int J Cancer* 2000;89(2):118-21. [https://doi.org/10.1002/\(SICI\)1097-0215\(20000320\)89:2<118::AID-IJC3>3.0.CO;2-8](https://doi.org/10.1002/(SICI)1097-0215(20000320)89:2<118::AID-IJC3>3.0.CO;2-8).
- Cheng G, Fan X, Hao M, Wang J, Zhou X, Sun X, et al. Higher levels of TIMP-1 expression are associated with a poor prognosis in triple-negative breast cancer. *Mol Cancer* 2016;15:30. <https://doi.org/10.1186/s12943-016-0515-5>.
- Castro NP, Chowdhury A, Jensen SM, Cuttitta F, Stetler-Stevenson WG, Solomon D. TIMP-2: A novel biologic therapy for triple negative breast cancer. *FASEB J* 2016;30(1):1144-54.
- Hammond ME, Hayes DF, Dowsett M, Allred DC, Hagerty KL, Badve S, et al. American society of clinical oncology/college of american pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer (unabridged version). *Arch Pathol Lab Med* 2010;134(7):e48-72.
- Lambert E, Dassé E, Haye B, Petitfrère E. TIMPs as multifacial proteins. *Crit Rev Oncol Hematol* 2004;49:187-98. <https://doi.org/10.1016/j.critrevonc.2003.09.008>.
- Radisky ES, Radisky DC. Matrix metalloproteinases as breast cancer drivers and therapeutic targets. *Front Biosci (Landmark Ed)* 2015;20:1144-63. <https://doi.org/10.2741/4364>.
- Vizoso FJ, González LO, Corte MD, Rodríguez JC, Vázquez J, Lamelas ML, et al. Study of matrix metalloproteinases and their inhibitors in breast cancer. *Br J Cancer* 2007;96(6):903-11. <https://doi.org/10.1038/sj.bjc.6603666>.
- Li HC, Cao DC, Liu Y, Hou YF, Wu J, Lu JS, et al. Prognostic value of matrix metalloproteinases (MMP-2 and MMP-9) in patients with lymph node-negative breast carcinoma. *Breast Cancer Res Treat* 2004;88(1):75-85. <https://doi.org/10.1007/s10549-004-1200-8>.

19. Talvensaari-Mattila A, Pääkkö P, Turpeenniemi-Hujanen T. Matrix metalloproteinase-2 (MMP-2) is associated with survival in breast carcinoma. *Br J Cancer* 2003;89(7):1270-5.
<https://doi.org/10.1038/sj.bjc.6601238>.
20. Pellikainen JM, Ropponen KM, Kataja VV, Kellokoski JK, Eskelinen MJ, Kosma VM, et al. Expression of matrix metalloproteinase (MMP)-2 and MMP-9 in breast cancer with a special reference to activator protein-2, HER2, and prognosis. *Clin Cancer Res* 2004;10(22):7621-8.
<https://doi.org/10.1158/1078-0432.CCR-04-1061>.
21. Roy DM, Walsh LA. Candidate prognostic markers in breast cancer: Focus on extracellular proteases and their inhibitors. *Breast Cancer (Dove Med Press)* 2014;6:81-91.
22. Bourboulia D, Stetler-Stevenson WG. Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs): Positive and negative regulators in tumor cell adhesion. *Semin Cancer Biol* 2010;20(3):161-8.
<https://doi.org/10.1016/j.semcancer.2010.05.002>.
23. Thorsen SB, Christensen SL, Würtz SO, Lundberg M, Nielsen BS, Vinther L, et al. Plasma levels of the MMP-9: TIMP-1 complex as prognostic biomarker in breast cancer: A retrospective study. *BMC Cancer* 2013;13:598.
<https://doi.org/10.1186/1471-2407-13-598>.
24. Lipton A, Ali SM, Leitzel K, Demers L, Evans DB, Hamer P, et al. Elevated plasma tissue inhibitor of metalloproteinase-1 level predicts decreased response and survival in metastatic breast cancer. *Cancer* 2007;109(10):1933-9.
25. Schrohl AS, Look PM, Gelder ME, Foekens JA, Brünner N. Tumor tissue levels of tissue inhibitor of Metalloproteinases-1 (TIMP-1) and outcome following adjuvant chemotherapy in premenopausal lymph node-positive breast cancer patients: A retrospective study. *BMC Cancer* 2009;9:322.
<https://doi.org/10.1186/1471-2407-9-322>.
26. Swellam M, Arab LR, Bushnak HA. Clinical implications of HER-2/neu overexpression and proteolytic activity imbalance in breast cancer. *IUBMB Life* 2007;59(6):394-401.
<https://doi.org/10.1080/15216540701395074>.
27. Brew K, Nagase H. The tissue inhibitors of metalloproteinases (TIMPs): An ancient family with structural and functional diversity. *Biochim Biophys Acta* 2010;1803(1):55-71.
<https://doi.org/10.1016/j.bbamcr.2010.01.003>.
28. Span PN, Lindberg RL, Manders P, Tjan-Heijnen VC, Heuvel JJ, Beex LV, et al. Tissue inhibitors of metalloproteinase expression in human breast cancer: TIMP-3 is associated with adjuvant endocrine therapy success. *J Pathol* 2004;202(4):395-402.
<https://doi.org/10.1002/path.1528>.
29. Nahleh Z. Androgen receptor as a target for the treatment of hormone receptor-negative breast cancer: An unchartered territory. *Future Oncol* 2008;4(1):15-21.
<https://doi.org/10.2217/14796694.4.1.15>.