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ORIGINAL ARTICLE

# The prognostic role of interleukin-8 (IL-8) and matrix metalloproteinases -2 and -9 in lymph node-negative untreated breast cancer patients

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## Summary

***Purpose:*** *To investigate the relationships, if any, between interleukin (IL) -8/matrix metalloproteinase (MMP)-2/MMP-9 and other prognostic variables in lymph node-negative untreated breast cancer patients, and to determine the prognostic value of these potential biomarkers.*

***Methods:*** *The study included 135 patients with known clinicopathological parameters. IL-8, MMP-2 and MMP-9 levels were determined by ELISA in primary tumor tissue lysates.*

***Results:*** *There were no significant relationships between IL-8/MMP-2/MMP-9 expression and available clinicopathological parameters (patient age, menopausal status, tumor size and tumor grade). Estrogen receptor (ER)$^-$ patients had higher levels of both IL-8 and MMP-9 (p=0.006 and p=0.04, respectively) compared to ER$^+$ patients; there was a significant negative correlation between ER and IL-8 (p=0.02). MMP-9 expression was significantly higher in patients with higher levels of IL-8 (p<0.001) and there was a significant positive correlation between IL-8 and MMP-9, as well as between progesterone receptor (PR) and MMP-2 (p<0.001 and p=0.05, respectively). PR$^+$ patients had higher levels of MMP-2 than PR$^-$ patients (p=0.03). Among the investigated biomarkers, only IL-8 had a statistically significant prognostic value in terms of relapse free survival (RFS) (p<0.001). Patients with higher levels of IL-8 had worse prognosis.*

***Conclusions:*** *Expression of IL-8 and consequently expression of MMP-9 could be hormonally regulated in breast cancer. IL-8 could be a marker of more aggressive, ER$^-$ breast cancer phenotype. Different expression of MMP-2 and MMP-9 regarding differential hormonal receptor expression could indicate distinct mechanisms of their regulation. It seems that IL-8 is a strong and independent unfavorable prognostic parameter in node-negative breast cancer. Node-negative patients with higher levels of IL-8 should be treated with adjuvant, especially IL-8 targeted therapy.*

***Key words:*** *breast cancer, estrogen receptor, interleukin 8, matrix metalloproteinase*

## Introduction

Patients with lymph node-negative breast carcinoma have more favorable prognosis compared to lymph node-positive breast carcinoma. Relapse following surgery is expected to 25-30% of node-negative patients, and only these patients would benefit from adjuvant therapy [1]. Currently, clinicopathological parameters are conventionally used as predictors of relapse, but they fail to classify patients accurately according to their potential clinical outcome. Therefore, great efforts have been made in recent years to identify new prognostic biomarkers that could be useful in defining node-negative breast cancer patients being in high risk for relapse.

IL-8 is a inflammatory cysteine-any amino acid-cysteine (CXC) chemokine originally discovered as a chemotactic factor for leukocytes. IL-8 contributes to human cancer progression in an autocrine and paracrine manner. Multiple mechanisms are involved in IL-8 action, including direct effects on angiogenesis, tumor cell growth and migration, and indirect effects via attracting host infiltration cells. Overexpression of IL-8 by tumor cells may lead to elevated infiltration of leuko-

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cytes, which have been shown to produce various growth factors, angiogenic factors and metalloproteinases. IL-8 can also function as a motility factor for tumor cells, which may be relevant to tumor invasion and development of metastasis [2,3].

MMPs are potent proteolytic enzymes that play a key role in degradation of basement membranes and the extracellular matrix (ECM) which is essential for tumor invasion and development of metastases. MMP-2 (gelatinase A) and MMP-9 (gelatinase B) cleave type IV collagen and gelatin (denatured collagen), which are the main structural components of the basement membrane [4]. MMP-2 and MMP-9 play an important role in the regulation of angiogenic factors as well as in the activation of growth factors and their receptors [5]. Overexpression of MMP-2 and MMP-9 has been implicated in the development and progression of many types of cancers [4]. In breast cancer, MMP-9 expression is upregulated in tumor-associated stromal cells, including neutrophils, macrophages and lymphocytes, and may play a role in tumor-associated inflammation [6].

The aim of the present study was to investigate the relationships, if any, between IL-8, MMP-2 and MMP-9 and other prognostic variables in lymph node-negative untreated breast cancer patients, with emphasis on their relation to steroid receptor status, and to determine the prognostic value of these potential biomarkers in terms of RFS.

## Methods

The study included 135 lymph node-negative primary breast cancer patients with known clinicopathological parameters. All patients underwent surgical removal of their primary tumor at the Institute of Oncology and Radiology of Serbia, Belgrade. After surgery, histological specimens were examined and classified according to the criteria of the AJCC/UICC (American Joint Committee on Cancer / Union International Contre le Cancer) for TNM stage and histological type. Age and menopausal status, regional lymph node sta¬tus (N), tumor size (T), tumor grade (G) and histological type were obtained after the Institutional Review Board approval. Postoperatively, none of the patients received adjuvant therapy due to favorable clinicopathological parameters, preferentially negative axillary lymph node status and absence of G3 tumor grade.

*IL-8, MMP-2 and MMP-9 estimation*

IL-8 levels were determined by ELISA in the primary tumor tissue lysates according to the manufacturer's instructions (RayBio Human IL-8 ELISA kit, USA). MMP-2 and MMP-9 levels were determined by ELISA in the primary tumor tissue lysates according to manufacturer's instructions (Quantikine Human MMP-2 Immunoassay, Quantikine Human MMP-9 Immunoassay, USA). Cut-off values for IL-8, MMP-2 and MMP-9 were selected according to observed median values (M=102.27 pg/mg, M=12.07 ng/mg, and M=1.85 ng/mg, respectively). The same primary tumor tissue lysates were used for steroid receptor determination using the standard DCC (dextran-coated-charcoal) assay. ER levels ≥ 10 fmol/mg and PR levels ≥ 20 fmol/mg were considered as positive.

*Statistics*

Survival curves for RFS were constructed according to the Kaplan-Meier method and compared with the log-rank test. The Mann-Whitney rank sum test was used to examine the distribution of quantitative IL-8, MMP-2 and MMP-9 values between different subgroups of patients according to clinicopathological parameters. The correlations between steroid receptors, IL-8, MMP-2 and MMP-9 were analyzed by the Spearman's rank order correlation test. A p-value less than 0.05 was considered as statistically significant.

## Results

The patient clinicopathological parameters at the time of primary diagnosis are shown in Table 1 and the patient distribution of quantitative IL-8, MMP-9 and MMP-2 values according to available clinicopathological parameters of patients in Table 2. The median follow-up time was 111 months. There were no significant differences (Mann-Whitney rank sum test) in the expression of IL-8/MMP-2/MMP-9 in the subgroups of patients according to clinicopathological parameters (patient age, menopausal status, tumor size and tumor grade).

There was a statistically significant difference in the quantitative IL-8 values between $ER^-$ and $ER^+$ subgroups of patients (Mann-Whitney rank sum test, p=0.006). $ER^-$ patients had higher levels of IL-8 (median value of IL-8 387.07 pg/mg) compared to $ER^+$ patients (IL-8 median value 91.16 pg/mg). A statistically significant negative correlation was found between ER and IL-8 (Spearman rank order test, p=0.02).

There was a statistically significant difference (Mann-Whitney rank sum test, p=0.04) in the quantitative MMP-9 values between $ER^-$ and $ER^+$ subgroups of patients. $ER^-$ patients had higher levels of MMP-9 (median value of MMP-9 4.62 ng/mg) than $ER^+$ patients (MMP-9 median value 1.79 ng/mg). There was a significant differ-

**Table 1.** Patient clinicopathological parameters at the time of primary diagnosis

| *Clinicopathological parameters* | *N* | *%* |
|---|---|---|
| Age (years) | | |
| ≤ 50 | 28 | 20.7 |
| > 50 | 107 | 79.3 |
| NA | 0 | 0 |
| Menopausal status | | |
| Premenopausal | 33 | 24.4 |
| Postmenopausal | 102 | 75.6 |
| NA | 0 | 0 |
| Estrogen receptor status | | |
| ER– | 40 | 29.6 |
| ER+ | 95 | 70.4 |
| NA | 0 | 0 |
| Progesterone receptor status | | |
| PR– | 106 | 78.5 |
| PR+ | 29 | 21.5 |
| NA | 0 | 0 |
| Tumor size (cm) | | |
| < 2 | 76 | 56.3 |
| ≥ 2 | 57 | 42.2 |
| NA | 2 | 1.5 |
| Histological type | | |
| IDC | 55 | 40.7 |
| ILC | 39 | 28.9 |
| Other histologies | 38 | 28.2 |
| NA | 3 | 2,2 |
| Grade | | |
| G1 | 14 | 10.4 |
| G2 | 116 | 85.9 |
| NA | 5 | 3.7 |

IDC: invasive ductal carcinoma, ILC: invasive lobular carcinoma, ER: estrogen receptor, PR: progesterone receptor, NA: data not available

ence (Mann-Whitney rank sum test, p<0.001) in the quantitative MMP-9 values between the subgroups of patients formed according to IL-8 median value (M=102.27 pg/mg). MMP-9 expression was significantly higher in patients with higher levels of IL-8 (MMP-9 median value 4.05 ng/mg) compared to patients with lower IL-8 levels (MMP-9 median value 1.05 ng/mg). A statistically significant positive correlation was found between IL-8 and MMP-9 (Spearman rank order test, p<0.001). MMP-9 expression was significantly higher (Mann-Whitney rank sum test, p=0.004) in patients with invasive ductal carcinoma (MMP-9 median value 3.37 ng/mg) compared to patients with invasive lobular carcinoma (MMP-9 median value 1.23 ng/mg).

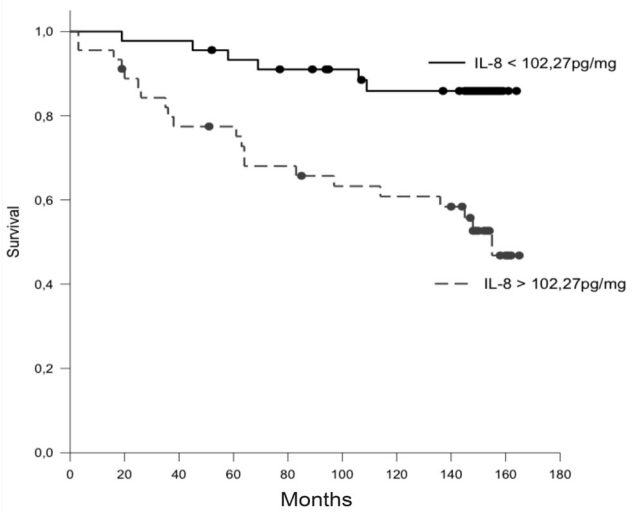


**Figure 1.** Survival analysis for subgroups of patients according to IL-8 median value (log rank, p<0.001).

There was a statistically significant difference (Mann-Whitney rank sum test, p=0.03) in the quantitative MMP-2 values between $PR^-$ and $PR^+$ subgroups of patients. $PR^+$ patients had higher levels of MMP-2 (median value of MMP-2 16.14 ng/mg) than $PR^-$ patients (MMP-2 median value 11.40 ng/mg). A statistically significant positive correlation was found between PR and MMP-2 (Spearman rank order test, p=0.05).

There was a statistically significant difference in RFS between subgroups of patients formed according to IL-8 median value (log rank test, p<0.001). Patients with IL-8 levels higher than the median value (M=102.27 pg/mg; range 5.07 – 1846.32) had worse prognosis (Figure 1). There was no statistically significant difference in RFS between subgroups of patients stratified according to MMP-9 median value (M=1.85 ng/mg; range 0.12 – 46.57 ng/mg), neither between subgroups of patients stratified according to MMP-2 median value (M = 12.07 ng/mg; range 1.85 – 49.84).

## Discussion

It is highly unlikely that a single biomarker will ever be able to act as an accurate prognosticator of disease outcome. It seems more probable that a widely accepted combination of different biomarkers will be used in the clinical decision-making in the near future. In the present study, we investigated the potential prognostic role of IL-8, MMP-2 and MMP-9 markers of invasiveness, in untreated node-negative breast cancer patients.

Among the investigated biomarkers, only IL-8 showed a statistically significant prognos-

**Table 2.** Distribution of quantitative IL-8, MMP-9 and MMP-2 values according to available clinicopathological parameters of the patients (Mann - Whitney rank sum test)

| *Clinicopathological parameters* | *N* | *Median IL-8 values (pg/mg)* | *p-value* | *N* | *Median MMP-9 values (ng/mg)* | *p-value* | *N* | *Median MMP-2 values (ng/mg)* | *p-value* |
|---|---|---|---|---|---|---|---|---|---|
| Age (years) | | | | | | | | | |
| ≤ 50 | 17 | 145.18 | 0.3 | 24 | 1.42 | 0.3 | 26 | 12.52 | 0.9 |
| > 50 | 73 | 95.80 | | 73 | 2.61 | | 86 | 11.69 | |
| Menopausal status | | | | | | | | | |
| Pre | 19 | 145.93 | 0.1 | 27 | 1.86 | 0.9 | 30 | 11.74 | 0.5 |
| Post | 71 | 94.55 | | 70 | 1.83 | | 82 | 12.07 | |
| Steroid receptor status | | | | | | | | | |
| ER– | 24 | 387.07 | 0.006 | 27 | 4.62 | 0.04 | 35 | 10.67 | 0.1 |
| ER+ | 66 | 91.16 | | 70 | 1.79 | | 77 | 12.25 | |
| PR– | 68 | 108.45 | 0.9 | 76 | 1.82 | 0.44 | 89 | 11.40 | 0.03 |
| PR+ | 22 | 91.84 | | 21 | 3.20 | | 23 | 16.14 | |
| Tumor size (cm) | | | | | | | | | |
| T < 2 | 50 | 88.24 | 0.1 | 59 | 1.61 | 0.1 | 67 | 12.02 | 0.8 |
| T ≥ 2 | 38 | 135.64 | | 37 | 2.85 | | 44 | 11.94 | |
| Histological type | | | | | | | | | |
| IDC | 35 | 107.55 | 0.08 | 42 | 3.37 | 0.004 | 48 | 12.71 | 0.3 |
| ILC | 26 | 92.62 | | 26 | 1.23 | | 34 | 12.09 | |
| Grade | | | | | | | | | |
| G1 | 9 | 53.55 | 0.4 | 13 | 1.43 | 0.2 | 13 | 11.64 | 0.5 |
| G2 | 78 | 106.41 | | 82 | 2.47 | | 96 | 12.07 | |

For abbreviations see footnote of Table 1

tic value. Patients with higher levels of IL-8 had worse prognosis (Figure 1). Recent studies have highlighted the prognostic and predictive significance of IL-8 in different types of cancer, including ovarian cancer [8], lung cancer [9], pancreatic cancer [10], colon cancer [11,12], bladder cancer [4,13], prostate cancer [14], leukemia [15], and melanoma [16]. IL-8 level was elevated in serum and tumor tissue of ovarian cancer patients, and increased IL-8 expression correlated with poor prognosis and survival [8]. The level of both IL-8 and MMP-9 in serum and tumor tissue of lung cancer patients was significantly higher compared to healthy controls and patients with benign respiratory diseases [9]. IL-8 was highly expressed in serum and tumor tissue of pancreatic cancer patients and patients with high serum levels of IL-8 should receive more active treatment due to the more aggressive behavior of their cancer [10]. Biasi et al. investigated the changes of blood levels of IL-8, MMP-9 and other biomarkers throughout the benign to malignant phases of colorectal neoplastic disease. IL-8 levels as well as MMP-9 activity and levels showed very similar trends, increasing concomitantly and only in patients with established cancer. Most likely, all such parameters drop dramatically after surgical removal of the malignant disease and may rise again in case of disease relapse [11]. In bladder cancer, higher expression of both IL-8 and MMP-9 in tumor tissue was related to unfavorable prognostic factors as well as to tumor recurrence [4]. IL-8 was identified as the most prominent urinary biomarker for the detection of bladder cancer [13]. IL-8 level was elevated in serum and tumor tissue of prostate cancer patients [14]. The expression of IL-8 and its receptors (CXCR1/CXCR2) in melanoma samples has the potential of becoming a biomarker of relative tumor aggressiveness [16]. In breast cancer patients, a positive relation has been established between increased serum IL-8 levels, advanced disease and poor survival [17-19]. In a study with the goal of identifying key factors involved in human breast cancer progression, human cytokine antibody arrays were used and IL-8 was identified as a key factor involved in breast cancer invasion and angiogenesis [20]. This is not surprising since recent studies have shown that the IL-8/CXCR1 signaling was important for breast cancer cell invasion [21] and was predominantly active in cells with the cancer stem cell phenotype [22,23]. Our results confirm that higher expression of IL-8 has negative prognostic significance for survival of breast cancer patients.

In our study $ER^-$ patients had higher levels of IL-8 than $ER^+$ patients, and also there was a significant negative correlation between ER and IL-8 levels. This is in agreement with recent studies that found IL-8 expression being inversely related to ER status in breast malignant tis-

sue sections [24-26] and breast cancer cell lines [20,27,28]. IL-8 overexpression in breast cancer cell lines involved a higher transcriptional activity of IL-8 gene promoter and required a complex cooperation between NF-kappaB, AP-1 and C/EBP transcription factors [29]. Also, it is possible that ER could down-regulate the promoter of the IL-8 gene. Generally, breast cancer cell lines expressing ER are of low invasive and metastatic potential and express very low levels of several CXC chemokines including IL-8, whereas $ER^-$ cell lines are invasive, metastatic, and express high levels of these chemokines [25]. CXC chemokines are mainly produced by $ER^-$ breast tumors and are globally overexpressed in breast cancer metastases [25,26]. Involvement of estrogen in the regulation of IL-8 is somewhat contradictory. A recent study showed that estradiol increased IL-8 secretion in normal human breast tissue as well as in $ER^+PR^+$ breast cancer *in vitro* and *in vivo* [30]. As the breast is a hormone responsive tissue, it is important to consider the influence of estrogen on the expression of different factors/biomarkers involved in invasiveness and metastasis, such as cytokines and proteases. It is well known that ER status is an important parameter in breast cancer management and that $ER^+$ breast cancer patients have better prognosis due to their responsiveness to adjuvant endocrine therapy. It is very important to determine the biological characteristics of $ER^-$ breast tumors since they could not be hormonally treated. Our results imply that expression of IL-8 could be hormonally regulated in breast cancer. Since there is increased expression of IL-8 in $ER^-$ breast tumors, IL-8 could be a marker of more aggressive, $ER^-$ breast cancer phenotype considering its association with tumor invasiveness, metastasis and poor prognosis. In order to deal with $ER^-$ breast tumors, IL-8 signaling could be one promising target.

The expression of the two gelatinases, MMP-2 and MMP-9, in breast cancer is well investigated in many studies using different methods. Although many studies have demonstrated prognostic and predictive significance of MMP-2 and/or MMP-9 in serum and tumor tissue of breast cancer patients [31-34], our results showed that neither MMP-2 nor MMP-9 had a significant prognostic value in terms of RFS. Patel et al. showed that serum MMP-9 level was a better marker than serum MMP-2 level in predicting breast cancer development and progression [31]. The enzymatic activity of urinary MMP-9/lipocalin-2 complex was detected in the urine of breast cancer patients but not in healthy controls [35]. A recent study demonstrated that elevated serum levels of lipocalin-2 and MMP-9 were associated with reduced disease free survival of breast cancer patients, particularly of patients with lower body mass index as well as lymph node-negative patients [36]. Several studies on breast tumor tissue sections showed that MMP-2 and/or MMP-9 were unfavorable prognostic parameters in lymph node-negative patients [1,7,37]. On the contrary, according to Scorilas et al. study on breast tumor tissue sections, MMP-9 was an independent favorable prognostic parameter in node-negative patients [38]. The differences between the results of these studies - including ours - might be due to the different detection methods used. Although MMP-2 and MMP-9 have indeed an important role in cancer progression, it must be taken into consideration that the matrix-degrading proteolytic activity of a tumor is the result of a complex balance between proMMPs, active MMPs, MMP activators and TIMPs, and the balance is self-controlled by interactions between tumor cells and host-derived stromal cells.

Our results showed that although neither of these MMPs had a significant prognostic value in node-negative breast cancer patients, their expression was significantly related to steroid receptor status. In our study, $ER^-$ patients had higher levels of MMP-9. This is in agreement with the study by La Rocca et al. [39] that demonstrated a significant inverse correlation between ER and MMP-9 levels, as well as between ER and MMP-2 levels in breast cancer sera. In addition, a study by Sullu et al. [40] on breast tumor tissue sections showed that expression of both MMP-2 and MMP-9 was significantly increased in $ER^-$ tumors. In another study on breast cancer cell lines, $ER^-$ cells expressed significantly more MMP-9 than $ER^+$ cells [41]. Estradiol treatment significantly decreased the activity of both MMP-2 and MMP-9 in human breast cancer *in vitro* and *in vivo* [42,43]. Estradiol induced a significant decrease of intracellular and secreted MMP-2 and MMP-9 levels in $ER^+PR^+$ human breast cancer cells [42]. Surprisingly, in our study $PR^+$ patients had higher levels of MMP-2 than $PR^-$ patients and also MMP-2 levels were in direct correlation with PR levels and lack of correlation with ER levels. As mentioned above, the aforementioned studies [39,40] showed negative relation between MMP-2 and ER status, but there is a lack of published data regarding possible relations between MMP-2 and PR expression, especially in breast cancer. Although the MMP-2

promoter lacks a canonical progesterone response element (PRE), progesterone inhibits MMP-2 expression and is part of treatment protocols in gynecological invasive pathologies, including endometriosis and endometrial hyperplasia. A study on human choriocarcinoma cell line demonstrated that progesterone significantly decreased the secretion of pro-MMP-2, as well as MMP-2 transcript expression level in a dose-dependent manner [44]. Even more, in human deciduas the effect of progesterone on MMP-2 expression is dependent on the expression of PR isoforms. Progesterone decreased MMP-2 expression in decidua with overexpressed PR-B, and increased MMP-2 expression in decidua with overexpressed PR-A or PR-C [45].

In our study MMP-9 expression was significantly higher in patients with higher levels of IL-8 vs those with lower IL-8 levels and also there was a significant positive correlation between IL-8 and MMP-9 levels. This is in agreement with studies that showed a significant positive correlation between IL-8 and MMP-9 levels in serum of colorectal cancer patients [11] as well as in tissue of lung cancer patients [9]. In addition, IL-8-enhanced ovarian cancer cell invasiveness correlated with increased MMP-2 and MMP-9 expression and activity [8]. These findings suggest a possible universal mechanism of invasiveness via IL-8. IL-8 influences induction of transcription of both MMP-2 and MMP-9 in tumor cells and endothelial cells, increasing stromal invasion by tumor cells, facilitating angiogenesis and development of metastasis in many types of cancer [46-48].

Our results imply that expression of IL-8 and consequently expression of MMP-9 could be hormonally regulated and that expression of MMP-9 is indeed regulated by IL-8. This could be a plausible explanation for a more aggressive biological behavior of $ER^-$ breast cancer. However, different expression of these two MMPs (MMP-2 regulated by PR and MMP-9 regulated by ER) regarding differential hormonal receptor expression, could indicate distinct mechanisms of their regulation. Moreover, it seems that IL-8 is a strong and independent unfavorable prognostic parameter in node-negative breast cancer. Owing to this, node-negative breast cancer patients with higher levels of IL-8 should be treated with adjuvant, especially IL-8 targeted therapy, such as neutralizing antibodies to CXCR1/CXCR2 receptors, a small-molecule CXCR1 inhibitor - repertaxin [22], and a humanized antibody against IL-8.

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