
Interleukin 8 in progression of hormone-dependent early breast cancer

JELENA MILOVANOVIC* , NATASA TODOROVIC-RAKOVIC, TIJANA VUJASINOVIC and ZAKI ABU RABI

Department of Experimental Oncology, Institute of Oncology and Radiology of Serbia, Belgrade, Serbia

**Corresponding author (Email, jelena.mil10@gmail.com)*

The only way to perceive the real clinical course of disease and the prognostic significance of potential biomarkers is follow-up of patients who did not receive any kind of adjuvant therapy. Many studies have confirmed high levels of interleukin 8 (IL8) in HER2-enriched and basal-like (ER⁻) primary breast tumours, but less is known about the significance of IL8 in hormone-dependent breast cancer. The aim of this study was to evaluate the prognostic significance of IL8 and clinicopathological parameters in hormone-dependent breast cancer, and to examine possible associations between them that might imply possible biological dependence. The study included 91 early-stage breast cancer patients with detectable levels of hormone receptors (ER>0, PR>0). None of the patients received adjuvant therapy according to valid protocol at that time. HER2 status was determined on paraffin-embedded tumour tissue sections by CISH. IL8 levels were determined by ELISA in cytosol tumour extracts of 65 patients with long-term follow-up (144 months). Nonparametric statistical tests were used for data analyses. Patients with low IL8 levels (M<88.8 pg/mg) had significantly longer relapse-free survival (RFS) compared to patients with high IL8 levels (M≥88.82 pg/mg) (Log rank test, p=0.002). Patients with ER^{high}IL8^{low} phenotype had significantly longer RFS compared to those with ER^{high}IL8^{high} and ER^{low}IL8^{high} phenotypes (p=0.04 and p=0.02, respectively); patients with PR^{low}IL8^{low} phenotype had significantly longer RFS compared to those with PR^{low}IL8^{high} and PR^{high}IL8^{high} phenotypes (p=0.003 and p=0.02, respectively); patients with HER2-IL8^{low} phenotype had significantly longer RFS compared to those with HER2-IL8^{high} and HER2+IL8^{high} phenotypes (p=0.01 and p=0.02, respectively). Our results indicate significant contribution of IL8 on survival of hormone-dependent early-stage breast cancer patients and association with established parameters such as ER/PR and HER2.

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1. Background

Breast cancer is hormone-dependent disease with great genetic, pathological and clinical heterogeneity. Hormone receptors (ER and PR) together with human epidermal growth factor receptor 2 (HER2) are the main determinants of breast cancer and still the most important established biomarkers. The only way to perceive the real clinical course of disease and the prognostic significance of potential biomarkers is follow-up of patients who did not receive any kind of adjuvant (postoperative) therapy and had only surgical intervention. Very little data is available regarding this

issue because some kind of adjuvant therapy is given to all breast cancer patients nowadays.

Interleukin 8 (IL8) has been intensively examined in recent years as potential prognostic biomarker in different types of human cancers. IL8 is an inflammatory cytokine that belongs to the class of CXC chemokines, a potent chemoattractant and activator of neutrophils, monocytes and other immune cells. Elevated expression of IL8 has been characterized in many different chronic inflammatory conditions and different types of human cancers (reviewed in Qazi *et al.* 2011). Literature have indicated that IL8 could contribute to tumour growth and progression directly, by

Keywords. Breast cancer; HER2; hormone receptor; interleukin 8

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

| Clinicopathological parameters | n | % |
|--------------------------------|----|------|
| Age (years) | | |
| <62 (median) | 44 | 48.4 |
| ≥62 | 47 | 51.6 |
| NA | 0 | 0 |
| Menopausal status | | |
| Premenopausal | 0 | 0 |
| Postmenopausal | 91 | 100 |
| NA | 0 | 0 |
| Tumour size (cm) | | |
| T<2 | 49 | 53.8 |
| T≥2 | 40 | 44.0 |
| NA | 2 | 2.2 |
| Nodal status | | |
| N0 | 91 | 100 |
| N+ | 0 | 0 |
| NA | 0 | 0 |
| Histological type | | |
| IDC | 36 | 39.5 |
| ILC | 27 | 29.7 |
| Other histologies | 26 | 28.6 |
| NA | 2 | 2.2 |
| Tumour grade | | |
| G1 | 9 | 9.9 |
| G2 | 80 | 87.9 |
| G3 | 1 | 1.1 |
| NA | 1 | 1.1 |
| Estrogen receptor status | | |
| ERlow | 20 | 22.0 |
| ERhigh | 71 | 78.0 |
| NA | 0 | 0 |
| Progesterone receptor status | | |
| PRlow | 70 | 76.9 |
| PRhigh | 21 | 23.1 |
| NA | 0 | 0 |
| HER2 status | | |
| HER2- | 65 | 71.4 |
| HER2+ | 20 | 22.0 |
| NA | 6 | 6.6 |
| Receptor status | | |
| ERlowPRlow | 20 | 22.0 |
| ERlowPRhigh | 0 | 0 |
| ERhighPRlow | 50 | 54.9 |
| ERhighPRhigh | 21 | 23.1 |
| NA | 0 | 0 |
| Receptor status | | |
| ERlowPRlowHER2- | 10 | 11.0 |
| ERhighPRhighHER2+ | 2 | 2.2 |
| NA | 6 | 6.6 |

n, number of patients; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; ERlow, ER <10 fmol/mg; ERhigh, ER ≥10 fmol/mg; PRlow, PR <20 fmol/mg; PRhigh, PR ≥20 fmol/mg; HER2-, HER2 gene not amplified; HER2+, HER2 gene amplified; NA, data not available.

acting as mitogenic and potent angiogenic factor, as well as indirectly, by chemotactic infiltration of immune cells that secrete by themselves growth and angiogenic factors (reviewed in Xie 2001). Moreover, recent studies have supported the involvement of IL8 signaling in regulation of stem cells activity, which is considered to be responsible for tumour initiation, maintenance, metastases and disease recurrence (Singh *et al.* 2013). Many studies have indicated that there are high levels of IL8 in basal-like (ER-) and HER2-enriched (HER2+) primary breast tumours, two subtypes characterized by a particularly poor prognosis (Aceto *et al.* 2012), but less is known about the role and the significance of IL8 in hormone-dependent breast cancer.

The aims of the study were to evaluate the prognostic significance of interleukin 8 and clinicopathological parameters in hormone-dependent breast cancer, and to examine possible associations between them that might imply possible biological dependence.

2. Materials and methods

The study included 91 early-stage breast cancer patients (clinical stadium I/II) with known clinicopathological parameters. All patients were postmenopausal and had detectable levels of hormone receptors (ER>0, PR>0) that should indicate hormone-dependent breast cancer. All patients underwent surgical removal of their primary tumour at the Institute of Oncology and Radiology of Serbia. After surgery, histological specimens were examined and classified according to the criteria of AJCC/UICC (American Joint Committee on Cancer/Union International Contre le Cancer) for TNM stage and histological type, and part of the tumour was frozen. Age and menopausal status, regional lymph node status (N), tumour size (T), tumour grade (G) and histological type were obtained after the Institutional Review Board approval. None of the patients received adjuvant therapy according to valid protocol at that time, due to favorable clinicopathological parameters, preferentially N0 status and absence of G3 tumour grade. The course of disease was followed from surgical intervention till relapse, so-called, relapse-free survival (RFS).

HER2 status (absence or presence of gene amplification) was determined on formalin-fixed paraffin-embedded tumour tissue sections by chromogenic in situ hybridization (CISH), according to the manufacturer's instructions (Invitrogen SPOT-Light HER2 CISH Kit, USA). Hybridization results were evaluated in 40× and 100× magnification (Olympus BX51 light microscope). One to 5 gene copies per nucleus were defined as no amplification, while more than 6 gene copies per nucleus or large gene copy clusters in more than 50% of tumour cells defined amplification.

Table 2. Survival analyses for subgroups of patients formed according to available clinicopathological parameters

| Clinicopathological parameters | Number of patients | Number of relapses | Relapses (%) | RFS (months) | Median RFS (months) | p-Value (Log Rank) |
|--------------------------------|--------------------|--------------------|--------------|--------------|---------------------|--------------------|
| Age (years) | | | | | | |
| <62 (median) | 44 | 12 | 28.6 | 4–161 | 95 | 0.5 |
| ≥62 | 47 | 11 | 23.9 | 3–165 | 127 | |
| NA | 0 | | | | | |
| Tumour size (cm) | | | | | | |
| T<2 | 49 | 8 | 16.3 | 3–161 | 144 | 0.03 |
| T≥2 | 40 | 14 | 35 | 3–165 | 95 | |
| NA | 2 | | | | | |
| Histological type | | | | | | |
| IDC | 36 | 5 | 13.9 | 6–165 | 145 | 0.01 |
| ILC | 27 | 11 | 40.7 | 3–158 | 69 | |
| NA | 2 | | | | | |
| Tumour grade | | | | | | |
| G1 | 9 | 1 | 11.1 | 24–158 | 145 | 0.3 |
| G2 | 80 | 22 | 27.5 | 3–165 | 103.5 | |
| NA | 1 | | | | | |
| ER status | | | | | | |
| ERlow | 20 | 5 | 25 | 3–162 | 65 | 0.6 |
| ERhigh | 71 | 18 | 25.3 | 3–165 | 108 | |
| NA | 0 | | | | | |
| PR status | | | | | | |
| PRlow | 70 | 19 | 27.1 | 3–165 | 106.5 | 0.5 |
| PRhigh | 21 | 4 | 19 | 4–165 | 97 | |
| NA | 0 | | | | | |
| Receptor status | | | | | | |
| ERlowPRlow | 20 | 5 | 25 | 3–162 | 65 | 0.5 |
| ERhighPRhigh | 21 | 4 | 19 | 4–165 | 97 | |
| NA | 0 | | | | | |
| HER2 status | | | | | | |
| HER2– | 65 | 16 | 24.6 | 3–165 | 114 | 0.8 |
| HER2+ | 20 | 5 | 25 | 10–155 | 101 | |
| NA | 6 | | | | | |

IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; ERlow, ER<10 fmol/mg; ERhigh, ER≥10 fmol/mg; PRlow, PR<20 fmol/mg; PRhigh, PR≥20 fmol/mg; HER2–, HER2 gene not amplified; HER2+, HER2 gene amplified; NA, data not available.

Table 3. Survival analysis for subgroups of patients formed according to the median IL8 level

| Biomarker | Number of patients* | Number of relapses | Relapses (%) | RFS (months) | Median RFS (months) | p-Value (Log Rank) |
|-----------|---------------------|--------------------|--------------|--------------|---------------------|--------------------|
| IL8 | | | | | | |
| IL8low | 33 | 5 | 15.1 | 19–161 | 150 | 0.002 |
| IL8high | 32 | 14 | 43.7 | 3–165 | 96 | |

*IL8 levels were measured in cytosol tumour extracts of 65 patients with long-term follow-up (144 months).

IL8low, IL8<88.82 pg/mg; IL8high, IL8≥88.82 pg/mg.

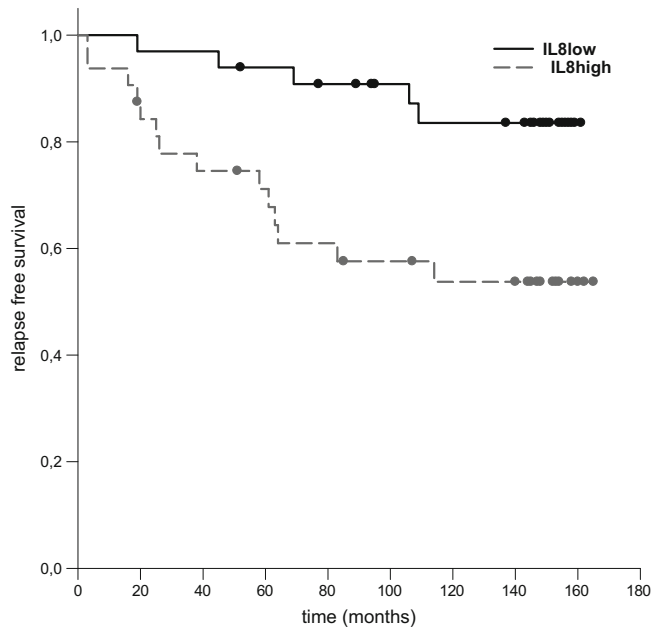


Figure 1. Survival analysis for subgroups of patients formed according to the median IL8 level (M=88.82 pg/mg).

Cytosol tumour extracts were prepared from frozen tumours by a following workflow: homogenization in 5 mM phosphate buffer pH 7.4–7.7 containing 20% glycerol, 1 mM monothioglycerol and 1.5 mM EDTA, centrifugation at 800–1000g for 30 min, followed by ultracentrifugation at

100000g for 60 min (ultracentrifuge Beckman L5-50). The whole procedure was performed at +4°C. Tumour extract protein concentrations were assayed by the Lowry method. Hormone receptor concentrations were determined by the standard ligand-binding assay. ER levels <10 fmol/mg and PR levels <20 fmol/mg was considered as low. ER levels ≥10 fmol/mg and PR levels ≥20 fmol/mg was considered as high.

IL8 concentrations were determined by ELISA in cytosol tumour extracts of 65 patients with long-term follow-up (144 months), according to the manufacturer's instructions (Ray-Bio® Human IL8 ELISA Kit, USA). The assay employs an antibody specific for human IL8 coated on a 96-well plate. IL8 present in the sample is bound to the well by immobilized antibody and colour develops in proportion to the amount of IL8 bound. The median IL8 value was used as cut off value.

2.1 Statistics

Survival curves for RFS were constructed according to the Kaplan-Meier method and compared with the log-rank test. Associations between parameters were analysed by the Chi-square test. The Mann-Whitney rank sum test was used to examine the distribution of quantitative IL8 levels between different subgroups of patients according to clinicopathological parameters. The correlations between steroid receptors and IL8 were analysed by the Spearman's rank order correlation test. A p-value less than 0.05 were considered as statistically significant.

Table 4. Survival analyses for subgroups of patients formed according to ER status and the median IL8 level

| Phenotype | Number of patients | Number of relapses | Relapses (%) | RFS (months) | Median RFS (months) | p-Value (Log Rank) |
|---------------|--------------------|--------------------|--------------|--------------|---------------------|--------------------|
| ERlowIL8low | 5 | 0 | 0 | 52–159 | 148 | 0.07 |
| ERlowIL8high | 9 | 5 | 55.5 | 3–165 | 114 | |
| ERhighIL8low | 28 | 5 | 17.9 | 19–161 | 150 | 0.04 |
| ERhighIL8high | 23 | 9 | 39.1 | 3–165 | 85 | |
| ERlowIL8low | 5 | 0 | 0 | 52–159 | 148 | 0.3 |
| ERhighIL8low | 28 | 5 | 17.9 | 19–161 | 150 | |
| ERlowIL8low | 5 | 0 | 0 | 52–159 | 148 | 0.1 |
| ERhighIL8high | 23 | 9 | 39.1 | 3–165 | 85 | |
| ERlowIL8high | 9 | 5 | 55.5 | 3–165 | 114 | 0.02 |
| ERhighIL8low | 28 | 5 | 17.9 | 19–161 | 150 | |
| ERlowIL8high | 9 | 5 | 55.5 | 3–165 | 114 | 0.5 |
| ERhighIL8high | 23 | 9 | 39.1 | 3–165 | 85 | |

ERlow, ER<10 fmol/mg; ERhigh, ER≥10 fmol/mg; IL8low, IL8<88.82 pg/mg; IL8high, IL8≥88.82 pg/mg.

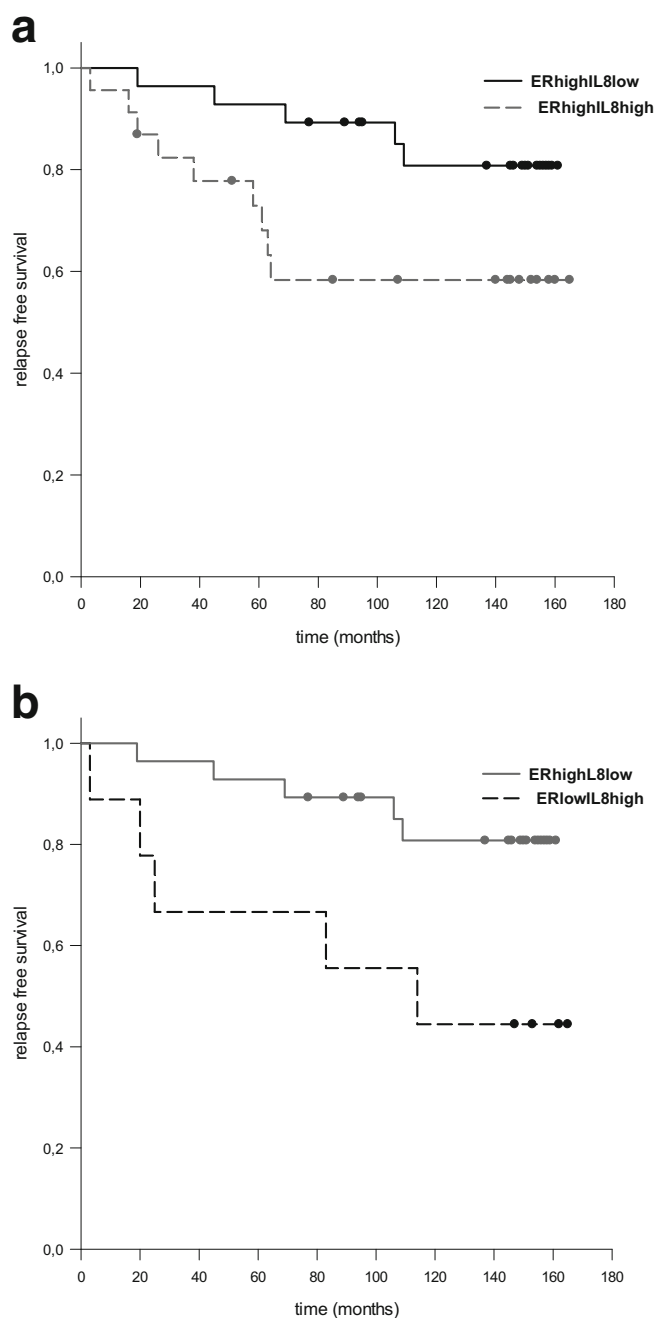


Figure 2. Survival analyses for subgroups of patients formed according to ER status and the median IL8 level ($M=88.82$ pg/mg).

3. Results

Patient's clinicopathological parameters at the time of primary diagnosis are presented in table 1. There were

no statistically significant differences in the expression of IL8 between subgroups of patients formed according to patient's age, tumour size, histological type, tumour grade and receptor status (Mann-Whitney rank sum test). There was no statistically significant correlation between ER and IL8 expression, neither between PR and IL8 expression (Spearman rank order test).

Survival analyses for subgroups of patients formed according to available clinicopathological parameters are presented in table 2. There were no statistically significant differences in relapse-free survival (RFS) between subgroups formed according to patient's age, tumour grade and receptor status but there was a statistically significant difference in RFS between subgroups formed according to tumour size (Log rank test, $p=0.03$) and histological type ($p=0.01$).

Survival analysis for subgroups formed according to the median IL8 value ($M=88.82$ pg/mg; range 5.07–1846.32 pg/mg) is presented in table 3. There was a statistically significant difference in RFS (Log rank test, $p=0.002$) and patients with low IL8 levels ($M<88.82$ pg/mg) had better prognosis (figure 1).

Survival analyses for subgroups of patients formed according to ER status and the median IL8 value ($M=88.82$ pg/mg) are presented in Table 4. In all combinations of phenotypes, patients with low IL8 levels ($M<88.82$ pg/mg) had lower percentage of relapses and longer RFS compared to patients with high IL8 levels ($M\geq 88.82$ pg/mg), regardless of ER status. There was a statistically significant difference in RFS between patients with ER^{high}IL8^{low} and ER^{high}IL8^{high} phenotypes (Log rank test, $p=0.04$) as well as between patients with ER^{high}IL8^{low} and ER^{low}IL8^{high} phenotypes ($p=0.02$). Patients with ER^{high}IL8^{low} phenotype had significantly longer RFS compared to patients with ER^{high}IL8^{high} phenotype (figure 2a) and patients with ER^{high}IL8^{low} phenotype had significantly longer RFS compared to patients with ER^{low}IL8^{high} phenotype (figure 2b).

Survival analyses for subgroups formed according to PR status and the median IL8 value ($M=88.82$ pg/mg) are presented in table 5. In all combinations of phenotypes, patients with low IL8 levels had lower percentage of relapses and longer RFS compared to patients with high IL8 levels, irrespective of PR status. There was a statistically significant difference in RFS between patients with PR^{low}IL8^{low} and PR^{low}IL8^{high} phenotypes ($p=0.003$) as well as between patients with PR^{low}IL8^{low} and PR^{high}IL8^{high} phenotypes ($p=0.02$). Patients with PR^{low}IL8^{low} phenotype had significantly longer RFS compared to patients with PR^{low}IL8^{high} phenotype

Table 5. Survival analyses for subgroups of patients formed according to PR status and the median IL8 level

| Phenotype | Number of patients | Number of relapses | Relapses (%) | RFS (months) | Median RFS (months) | p-Value (Log Rank) |
|---------------|--------------------|--------------------|--------------|--------------|---------------------|--------------------|
| PRlowIL8low | 24 | 2 | 8.3 | 45–161 | 150 | 0.003 |
| PRlowIL8high | 25 | 11 | 44 | 3–165 | 107 | |
| PRhighIL8low | 9 | 3 | 33.3 | 19–161 | 109 | 0.6 |
| PRhighIL8high | 7 | 3 | 42.9 | 16–160 | 85 | |
| PRlowIL8low | 24 | 2 | 8.3 | 45–161 | 150 | 0.07 |
| PRhighIL8low | 9 | 3 | 33.3 | 19–161 | 109 | |
| PRlowIL8low | 24 | 2 | 8.3 | 45–161 | 150 | 0.02 |
| PRhighIL8high | 7 | 3 | 42.9 | 16–160 | 85 | |
| PRlowIL8high | 25 | 11 | 44 | 3–165 | 107 | 0.5 |
| PRhighIL8low | 9 | 3 | 33.3 | 19–161 | 109 | |
| PRlowIL8high | 25 | 11 | 44 | 3–165 | 107 | 0.8 |
| PRhighIL8high | 7 | 3 | 42.9 | 16–160 | 85 | |

PRlow, PR<20 fmol/mg; PRhigh, PR≥20 fmol/mg; IL8low, IL8<88.82 pg/mg; IL8high, IL8≥88.82 pg/mg.

(figure 3a) and patients with PRlowIL8low phenotype had significantly longer RFS compared to patients with PRhighIL8high phenotype (figure 3b).

Survival analyses for subgroups formed according to HER2 status and the median IL8 value (M=88.82 pg/mg) are presented in table 6. In all combinations of phenotypes, patients with low IL8 levels had lower percentage of relapses and longer RFS compared to patients with high IL8 levels, regardless of HER2 status. There was a statistically significant difference in RFS between patients with HER2–IL8low and HER2–IL8high phenotypes (p=0.01) as well as between patients with HER2–IL8low and HER2+IL8high phenotypes (p=0.02). Patients with HER2–IL8low phenotype had significantly longer RFS compared to patients with HER2–IL8high phenotype (figure 4a) and patients with HER2–IL8low phenotype had significantly longer RFS compared to patients with HER2+IL8high phenotype (figure 4b).

4. Discussion

Our results showed prognostic significance of IL8 and patients with low IL8 levels had better prognosis (figure 1). Recent literature have indicated the significance of IL8 as potential prognostic marker in different types of human cancers. Increased IL8 expression in ovarian cyst fluid, ascites, serum and tumour tissue from ovarian cancer patients correlated with poor prognosis and survival (Wang *et al.* 2012). Increased IL8 expression in tumour tissue from endometrial cancer patients was associated with poor survival (Mannelqvist *et al.* 2011). Two large clinical studies showed that increased levels of both serum IL6 and IL8 were associated with lung cancer, but only IL8 levels were in direct relation to lung cancer risk

several years before diagnosis (Pine *et al.* 2011). Some authors indicated that IL8 could be a marker for predicting the prognosis and monitoring disease progression of pancreatic cancer patients (Chen *et al.* 2012) as well as colorectal cancer patients (Biasi *et al.* 2012). Reis *et al.* first indicated the prognostic relevance of IL8 mRNA expression in bladder cancer (Reis *et al.* 2012). Nasopharyngeal carcinoma (NPC) has the highest metastatic potential among head and neck cancers. A study on 250 patients with NPC revealed that higher IL8 expression in primary tumour tissue was an independent prognostic factor for overall survival, disease-free survival and distant metastasis-free survival (Li *et al.* 2012).

Literature related to the prognostic significance of IL8 in early and advanced breast cancer is not extensive. Skoog *et al.* showed that inflammatory breast cancer, a distinct and aggressive form of locally advanced breast cancer, was associated with high levels of IL8 (Skoog *et al.* 1987). According to literature, IL8 from serum and/or tumour tissue could be used to diagnose women with breast cancer and to identify patients with poor prognosis (Kozłowski *et al.* 2003; Benoy *et al.* 2004; Yao *et al.* 2007). Benoy *et al.* showed that serum IL8 had an independent prognostic significance for postrelapse survival (Benoy *et al.* 2004) and Yao *et al.* showed significance of tumour tissue IL8 for disease-free survival (Yao *et al.* 2007). Papaxoinis *et al.* showed that HER2-ECD, NO and IL8 plasma levels had prognostic value for survival of patients with metastatic breast cancer treated with weekly docetaxel (Papaxoinis *et al.* 2010). High pretreatment IL8 levels were of adverse prognostic value for survival and patients with high IL8 relative change had worse progression-free survival. Two large clinical studies showed that the polymorphisms in IL8 and CXCR2 genes

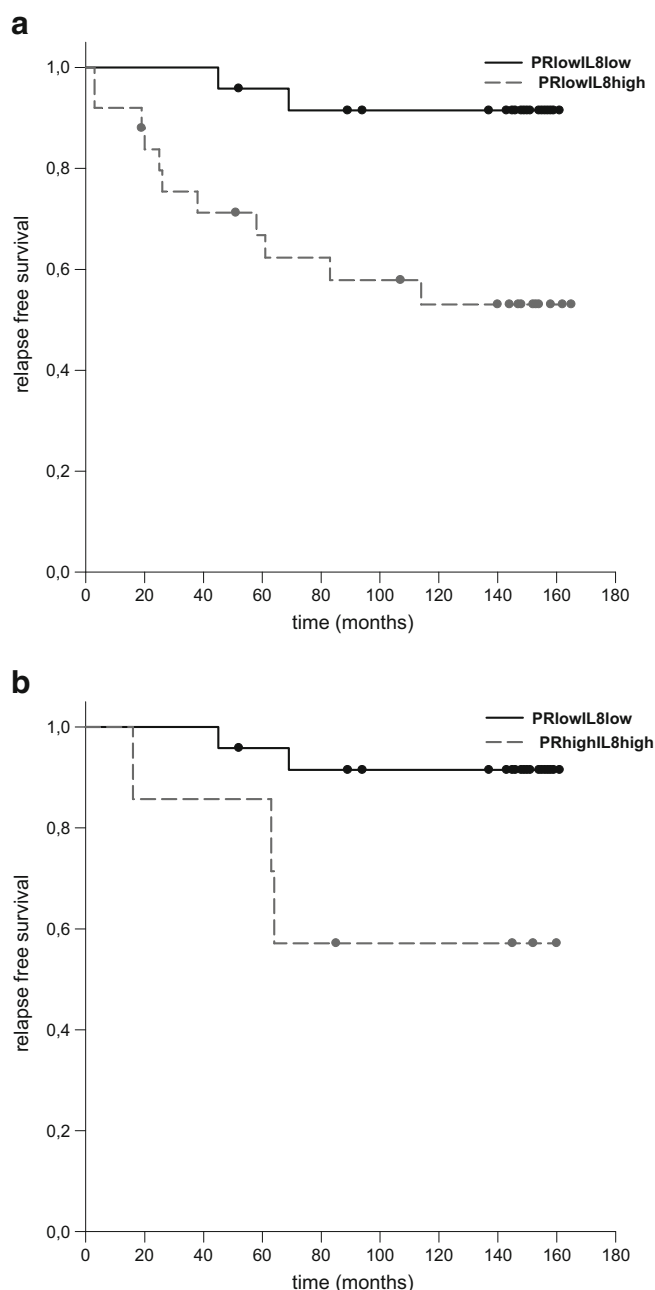


Figure 3. Survival analyses for subgroups of patients formed according to PR status and the median IL8 level ($M=88.82$ pg/mg).

were associated with increased breast cancer risk and prognosis (Snoussi *et al.* 2010). The presence of the IL8 (-251) A allele and/or the CXCR2 (+1208) T allele showed a significant association with decreased overall survival and decreased disease-free survival. We have recently shown the prognostic relevance of IL8 in adjuvantly untreated early breast cancer patients in short-term

and long-term follow-up (Milovanović *et al.* 2013; Abu Rabi *et al.* 2015).

The most important relation of IL8 is to ER and/or HER2 status, since receptor status is still the main determinant of breast cancer. According to literature, IL8 is highly expressed in ER- breast cancers and it is also highly expressed in HER2-enriched breast cancers (Freund *et al.* 2003; Yao *et al.* 2007; Aceto *et al.* 2012). Moreover, IL8 expression is inversely related to ER status in breast cancer cells (Freund *et al.* 2003; Aceto *et al.* 2012). It is postulated that the net effect of IL8 action in breast cancer is the result of a delicate balance between ER inhibition and HER2 induction of IL8 expression and activity (reviewed in Todorović-Raković and Milovanović 2013). In our study receptor status did not have prognostic significance (table 2), although available literature have implied that hormone receptor-positive and/or HER2-negative status is generally associated with better prognosis (Clark *et al.* 1983; Slamon *et al.* 1987; Dunnwald *et al.* 2007).

According to our results, IL8 seems to be more reliable prognostic marker than receptor status in early-stage breast cancer. When comparing RFS of patients stratified in different phenotypes according to receptor status and the median IL8 value (tables 4, 5 and 6), in all combinations of phenotypes patients with low IL8 levels had lower percentage of relapses and longer RFS compared to patients with high IL8 levels, irrespective of ER/PR/HER2 status.

When patients were stratified according to ER status and the median IL8 level (table 4), IL8 expression had no influence on survival of ERlow subgroup, but had significant influence on survival of ERhigh subgroup. Patients with ERhighIL8low phenotype had almost twice longer median RFS (150 months) compared to patients with ERhighIL8high phenotype (85 months) and the number of relapses in ERhighIL8low subgroup was 18% versus 39% in ERhighIL8high subgroup. This implies the significant role of IL8 in progression of hormone-dependent breast cancer, in contrast to the previously accepted hypothesis that IL8 expression is important feature that exclusively influences the course of disease of ER- breast cancer. Furthermore, patients with ERhighIL8low phenotype had significantly longer median RFS compared to patients with ERlowIL8high phenotype (150 and 114 months, respectively). This indicates that ER as a marker of favorable prognosis and IL8 as a marker of unfavorable prognosis (which is probably downregulated by ER), could influence the course of disease in an additive manner. It is interesting that according to our results, the impact of IL8 on survival is much stronger in PRlow tumours compared to PRhigh tumours (table 5). This

Table 6. Survival analyses for subgroups of patients formed according to HER2 status and the median IL8 level

| Phenotype | Number of patients | Number of relapses | Relapses (%) | RFS (months) | Median RFS (months) | p-Value (Log Rank) |
|--------------|--------------------|--------------------|--------------|--------------|---------------------|--------------------|
| HER2–IL8low | 27 | 4 | 14.8 | 19–161 | 150 | 0.01 |
| HER2–IL8high | 19 | 8 | 42.1 | 3–165 | 64 | |
| HER2+IL8low | 6 | 1 | 16.7 | 45–158 | 153 | 0.2 |
| HER2+IL8high | 8 | 4 | 50 | 3–158 | 100 | |
| HER2–IL8low | 27 | 4 | 14.8 | 19–161 | 150 | 0.9 |
| HER2+IL8low | 6 | 1 | 16.7 | 45–158 | 153 | |
| HER2–IL8low | 27 | 4 | 14.8 | 19–161 | 150 | 0.02 |
| HER2+IL8high | 8 | 4 | 50 | 3–158 | 100 | |
| HER2–IL8high | 19 | 8 | 42.1 | 3–165 | 64 | 0.2 |
| HER2+IL8low | 6 | 1 | 16.7 | 45–158 | 153 | |
| HER2–IL8high | 19 | 8 | 42.1 | 3–165 | 64 | 0.7 |
| HER2+IL8high | 8 | 4 | 50 | 3–158 | 100 | |

HER2–, HER2 gene not amplified; HER2+, HER2 gene amplified; IL8low, IL8 < 88.82 pg/mg; IL8high, IL8 ≥ 88.82 pg/mg.

could be explained by the fact that the most frequent subtype of tumours in this study was ERhighPRlow (55%).

When patients were stratified according to HER2 status and the median IL8 level (table 6), IL8 expression had significant influence on survival of HER2–subgroup, but had no influence on survival of HER2+ subgroup. Patients with HER2–IL8low phenotype had significantly longer median RFS compared to patients with HER2–IL8high phenotype (150 and 64 months, respectively) and the number of relapses in HER2–IL8low subgroup was 15% versus 42% in HER2–IL8high subgroup. Although IL8 has been identified as a marker of HER2-enriched breast cancer, our results indicate the significant influence of IL8 on the course of disease of HER2–breast cancer. Furthermore, patients with HER2–IL8low phenotype had significantly longer median RFS compared to patients with HER2+IL8high phenotype (150 and 100 months, respectively). This implies that HER2 and IL8 as markers of unfavorable prognosis, could act additively on the course of disease.

According to our results, if IL8 is highly expressed, the influence of ER is weaker and that is the reason why there is no significant difference in RFS between patients with ERlowIL8high and ERhighIL8high phenotypes. The same is true for PR and HER2 and there is no significant difference in RFS between patients with PRlowIL8high and PRhighIL8high phenotypes, neither between patients with HER2–IL8high and HER2+IL8high phenotypes.

Literature related to the hormonal regulation of IL8 is controversial. Many authors found that multiple cytokines including IL8 were overexpressed in ER– breast cancer (Bièche *et al.* 2007; Chavey *et al.* 2007). On the other

hand, Bendrik and Dabrosin found a significant positive correlation between IL8 and estradiol in normal breast tissue *in situ* and ER+PR+ breast cancer *in vivo* (Bendrik and Dabrosin 2009). Wen *et al.* first demonstrated *in vitro* and in mice *in vivo* that expression of IL8 was directly stimulated by activation of HER2 and downstream PI3K/Akt pathway (Wen *et al.* 2006). Azenshtein *et al.* showed that epidermal growth factor (HER2 ligand) potently upregulated IL8 secretion by breast tumour cells, and its effect was promoted by a consecutive treatment of the cells by estrogen and progesterone (Azenshtein *et al.* 2005). Haim *et al.* showed that EGF and estrogen upregulated in an additive manner the transcription and the secretion of IL8 in breast tumour cells (Haim *et al.* 2011). They postulated that EGF and estrogen probably act in independent transcriptional pathways that complement each other, in the upregulation of IL8.

Our results indicate significant contribution of IL8 on survival of hormonal dependent early-stage breast cancer patients and association with established parameters such as ER/PR and HER2. However, we must refrain and point that these results deserve further investigation, since we had small and unequal numbers of observations in subgroups. Our results are in contrast to the previously accepted hypothesis that IL8 expression is important feature that exclusively influences the course of ER– breast cancer. Furthermore, although IL8 has been identified as a marker of HER2-enriched breast cancer, our results indicate the significant influence of IL8 on the course of HER2–breast cancer. Moreover, not only expression of IL8 is dependent on ER and HER2 cross-talk, but also receptor-mediated signaling could act additively with IL8 in progression of hormone-dependent breast cancer.

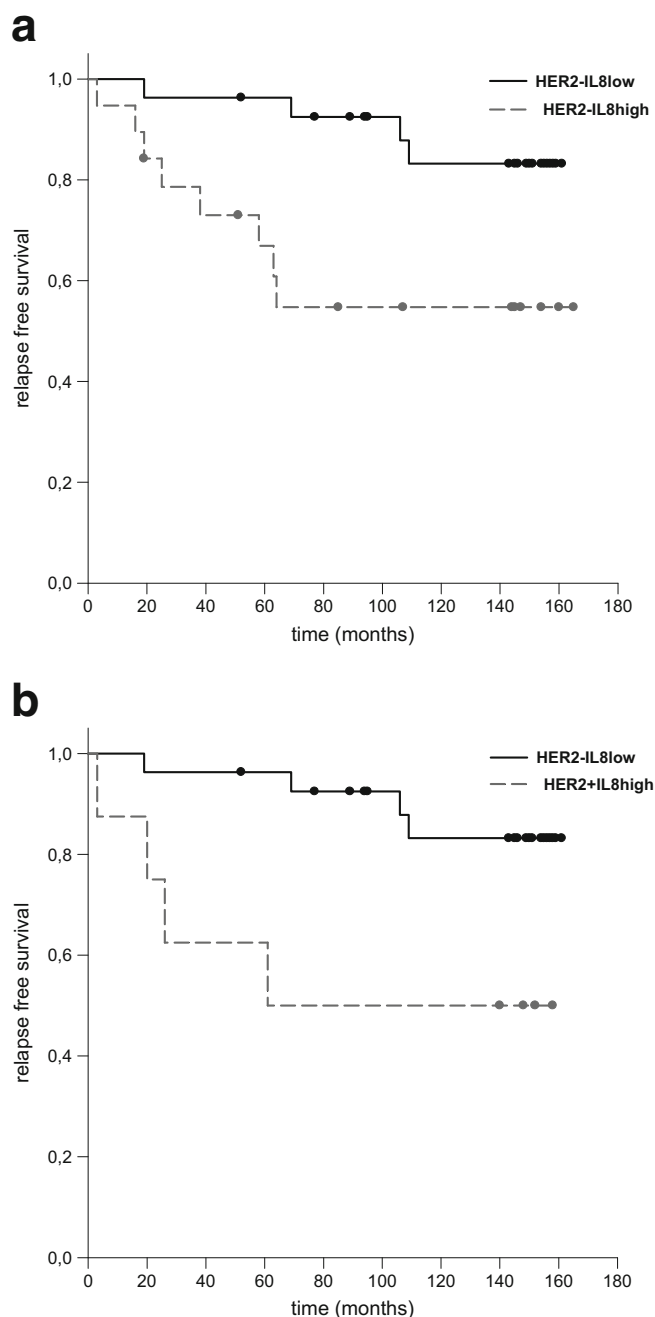


Figure 4. Survival analyses for subgroups of patients formed according to HER2 status and the median IL8 level ($M=88.82$ pg/mg).

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