**Materials and Methods:** A triple-negative breast cancer cell line (GPR30−1) was established from a clinical specimen under an IRB-approved tumor banking protocol. GPR30 expression in this cell line was demonstrated by immunohistochemical staining and RTPCR. The effect of GPR30 knock-down was assayed on GPR30−1 cells that were transfected with a 29mer short hairpin RNA (shRNA) constructed against GPR30 (Origene Technologies, Inc, Rockville, MD). Control cells were transfected with a non-effective 29mer shRNA cassette. Transient expression of green fluorescence protein (GFP) allowed selection of transfected cells by fluorescence-activated cell sorting (FACS). Proliferation of untransfected GPR30−1, control, and GPR30 knock-down cells was tested in normal medium, 20 and 100 micromolar TAM using the MTS assay.

**Results:** Control-transfected GPR30−1 cells had an equal proliferation rate to untransfected cells in normal medium (ratio 1.04:1.0). In normal medium, GPR30-knockdown cells had a reduced proliferation rate (ratio 0.34:1.0) compared to negative control or untransfected cells. Untransfected and control-transfected GPR30−1 cells showed a 53% and 43% decrease in proliferation after 24 hours in low-dose (20 micromolar) TAM compared to a 92% reduction in GPR30-knockdown cells. All cells showed a greater than 90% decrease in proliferation in high-dose (100 micromolar) TAM.

**Conclusions:** Knock-down of GPR30 in GPR30−1 cells lowers the proliferation of cells in normal medium and when exposed to TAM compared to normal and control-transfected cells. These results are consistent with a tamoxifen resistance effect of GPR30. GPR30 may represent a therapeutic target in breast cancer.

**181** Poster

**Cytotoxicity of docetaxel, epirubicin and carboplatin on hormonal receptors positive and triple negative breast cancer cell lines**

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**Background:** Breast cancer patients are stratified into three main groups: those expressing hormonal receptors (HR), which respond to therapies targeting estrogen receptors; HER2 positive tumors that are candidate for targeting therapy with trastuzumab or lapatinib; triple negative (TN) tumors, for which the only systemic therapy available is standard chemotherapy. Some studies suggest an increase susceptibility of TN to platinum-derived chemotherapy, with a pathological remission rate of 21%.

The purpose of this study was to evaluate cytotoxic capacity of docetaxel, epirubicin and carboplatin in MCF7 (HR positive) and HCC 1806 (TN) breast cancer cell lines.

**Material and Methods:** Human breast cancer cell lines MCF7 and HCC1806 were purchased to ATCC and cultured according to recomended procedures. Both cell lines were incubated in absence and presence of the docetaxel, epirubicin and carboplatin in several concentrations ranging from 50nM to 150μM. The sensitivity of the cell lines to the drugs studied was analyzed using the MTT colorimetric assay, performed 24, 48 and 72 hours after incubation. Cytotoxicity was expressed as the percentage of inhibition of cell proliferation correlated with untreated cultures. Dose-response curves were established and the half maximal inhibitory concentration (IC50) was calculated in Origin7 software.

**Results:** Epirubicin on HCC 1806 at 24h had a higher IC50 than on MCF7 (2.3 *vs.* 1.8μM), but this performance reached a similar level at 72h. Focusing on docetaxel, IC50 was higher on MCF7 than on HCC 1806, showing a better performance considering cell death for HCC 1806. Considering carboplatin, the IC50 was considerably elevated on MCF7. In HCC 1806, carboplatin proved IC 50 of 44.8 μM and 8.6 μM at 48 and 72h, respectively.

Comparing epirubicin *vs.* carboplatin cytotoxicity on MCF7, IC50 was always high in carboplatin studies, with IC50 of 53.5 μM at 72h for these particular cells. Also on HCC 1806, carboplatin showed a worse activity than epirubin, emphasized by higher IC50 for carboplatin at 48h (44.9 μM) and 72h (8.6 μM) than epirubicin.

**Conclusions:** Epirubicin had a similar effect on HR positive and TN cell lines. On the contrary, docetaxel proved a better performance on TN than on HR positive cells, the last showing elevated IC50. Carboplatin reached less cytotoxicity than epirubicin either in HR positive and TN breast cancer cell lines.

**182** Poster

**Long-term effect of fulvestrant on hormone receptors and proliferation marker in breast cancer**

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**Background:** Fulvestrant has been shown in short-term (maximum of 4 months) pre-surgical studies of breast cancer to decrease expression of estrogen receptor (ER), progesterone receptor (PgR) and proliferation marker, Ki67. We present changes in these markers from 6 months and beyond (median time to progression of 25.8 months) in breast cancer patients treated with fulvestrant.

**Materials and Methods:** 32 post-menopausal women with locally advanced (n = 22) or metastatic breast cancer (n = 10) with measurable breast lesions had fulvestrant (250 mg. intra-muscularly monthly) as first-line primary endocrine therapy. Immunohistochemistry was performed on sequential breast tumour biopsies taken at diagnosis (before commencing fulvestrant, T1), 6 weeks (T2), 6 months (T3) and at progression (T4) of disease.

**Results:** Wilcoxon signed rank sum analysis revealed decrease in the levels of all 3 markers at all subsequent time-points from pre-treatment level (significance at $p < 0.05$) (table).

| Marker | Median level at T1 (range) | p-value for change | | |
|---|---|---|---|---|
| | | T1−T2 | T1−T3 | T1−T4 |
| ER H score | 130 (60–190) | <0.001 | <0.001 | 0.001 |
| PgR H score | 30 (0–270) | Non-significant | 0.001 | 0.012 |
| Ki67% stain | 18 (1–60) | 0.001 | <0.001 | 0.028 |

There was non-significant recovery of ER and Ki67 at progression (T4) compared with 6 months level (T3). Kaplan-Meier analysis revealed lower pre-treatment (T1) Ki67 predictive of longer TTP but no similar relation was noted with ER and PgR. While not apparent at T1, higher PgR at 6 weeks (T2) predicted for longer TTP ($p = 0.008$).

**Conclusions:** The remaining expression of ER (partly due to some recovery) may contribute to acquired resistance as ER is still available for cross-talk with growth factors. However, lack of total depletion of ER at progression on fulvestrant may also explain known clinical response to further endocrine therapies. Mix of low and high PgR cases within the responders in this series (median PgR of 30) suggests fulvestrant activity being largely dependent on ER irrespective of PgR. This study confirms decrease in Ki67 (and ER and PgR) expression seen earlier in literature but is the first study which shows statistically significant decrease beyond a median of 2 years.

**183** Poster

**A DNA signature to identify high-risk small node-negative breast cancer patients**

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**Purpose:** To identify a DNA signature to predict metastasis of small node-negative breast carcinoma

**Experimental Design:** The authors used Comparative Genomic Hybridization (CGH) array to analyze 168 pT1T2pN0 invasive ductal carcinoma patients with either good (no event 5 years after diagnosis: 111 patients) or poor (57 patients with early onset metastasis) outcome. A CGH classifier, identifying low and high-risk groups of metastatic recurrence, was established in a training set of 78 patients. This classifier was based on both genomic regions with statistically different alterations between the two groups of clinical outcome and the number of alterations. It was then tested on a validation set of 90 patients and compared to clinicopathological parameters.

**Results:** The genomic status of regions located on chromosomes 2p22.2, 3p23 and 8q21−24 and the number of segmental alterations were defined in the training set to classify tumors into low or high-risk groups. In the validation set, this CGH classifier produced a highly significant odds ratio of 10.39 (95% CI: 3.75–28.78, $p = 6.63 \times 10^{-6}$, Wald test) in univariate analysis with a sensitivity of 66%, a specificity of 84% and an accuracy rate of 78%. The 5-year metastasis-free survival analysis showed a highly significant difference between the two predicted groups (Hazard Ratio = 5.7, $p = 1.82 \times 10^{-7}$, log-rank test). Together with estrogen receptor and grade, this CGH classifier provided significant prognostic information in multivariate analysis.

**Conclusions:** In addition to classical parameters, this DNA signature may constitute an accurate tool to identify patients with T1T2N0 luminal tumors, who may benefit from adjuvant treatments.

EG, GP, AV-S, XS-G, BA and OD contributed equally.

**184** Poster

**HER-2/neu expression in T1 to T3 breast cancer with extracapsular extension of axillary lymph node metastasis**

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**Background:** Studies on the association of HER-2/neu with the axillary lymph node metastasis are controversial. Amplification of the protein product of the HER-2/neu oncogene in primary breast cancer specimens is associated with an adverse prognosis.

**Methods:** From January 2000 to January 2009, 504 breast cancer patients operated in General hospital “Sveti Vracevi” in Bijeljina. We selected 253 (50.2%) patients with breast cancer who had metastases to axillary lymph nodes.

**Results:** Extracapsular extension (ECM) was found in 103 (40.7%). The patients were identified and divided into two groups: patients in the HER-2 positive group (38 patients) and HER-2 negative group (65 patients). In the HER-2 positive group ECM was seen in 62.5% patients compared with 37.4% in the HER-2 negative group (P = 0.059). 41 patients (39.8%) were identified with three or less lymph nodes involved, 30 patients (29.1%) patients four to six, 20 patients (19.4%) seven to nine, and 11 patients (10.6%) ten or more nodes, respectively. Total number of lymph nodes showing ECM were also significantly more in the HER-2 positive group (48 of 81, 59.25%) *vs.* (13 of 60, 21.66%) in the HER-2 negative group ($P < 0.001$). With a median follow-up of 96 months factors with independent prognostic value for disease-free survival by multivariate analysis included HER-2/neu overexpression with extracapsular extension ($P < 0.005$), pN category ($P < 0.01$), presence of lymphovascular invasion (LVI; $P < 0.005$), and ECM ($P < 0.001$). An independent negative prognostic effect on overall survival was observed for HER-2/neu overexpression with extracapsular extension ($P < 0.05$), pN category ($P < 0.05$), and presence of LVI ($P < 0.005$) and ECM ($P < 0.001$).

**Conclusions:** In patients whose tumors expressed HER-2/neu who had positive lymph nodes and extracapsular extension prognosis was significantly worse compared with those who were HER-2/neu negative and lymph node positive with extracapsular extension. These findings have led to the conclusion that HER-2/neu overexpression is associated with a more aggressive subtype of cancer.

**185** Poster

**TNF superfamily gene polymorphism as prognostic factor in early breast cancer**

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**Purpose:** Since apoptosis may play a role in the prognosis of breast cancer, the present study analyzed the polymorphisms of apoptosis-related genes and their impact on the survival of 240 patients with early invasive ductal breast cancer.

**Methods:** The genomic DNA was extracted from paraffin-embedded tumor-free tissue or blood, and 12 single nucleotide polymorphisms (SNPs) of 11 apoptosis-related genes in the apoptosis pathway determined using a Sequenom MassARRAY system.

**Results:** During the median follow-up of 53.4 (range, 2.9–205.9) months, 37 relapses and 22 deaths occurred. Among the target polymorphisms, the tumor necrosis factor superfamily member 10 gene polymorphism (TNFSF10 rs1131532) in a recessive model of the T allele and prostaglandin-endoperoxide synthase 2 gene polymorphism (PTGS2 rs5275) in a dominant model of the C allele were associated with survival in a log-rank test. The TT genotype of TNFSF10 (rs1131532) was also significantly correlated with a lower disease-free, distant disease-free, and overall survival in a multivariate analysis (HR = 3.304, 4.757, and 6.459; $P = 0.002$, 0.001, and 0.009, respectively), while PTGS2 rs5275 was only associated with a higher distant disease-free survival (HR = 0.302; $P = 0.041$). No clinicopathologic difference was observed according to the genotypes of these two polymorphisms.

**Conclusion:** The TNFSF10 (rs1131532) polymorphism was identified as a possible prognostic factor of survival in patients with operated invasive breast cancer.

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**Reference gene selection to quantify urokinase plasminogen activator in breast cancer**

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**Background:** Cancer biomarker research has been improved using real-time PCR. Normalization of target gene expression to a control gene is a common way to quantify gene expression changes. Some housekeeping genes have been used widely for quantification. Several studies show that the expression of some housekeeping genes alters in breast cancer tissues; hence they are not suitable for gene expression analysis. Therefore finding stable genes will help to investigate gene expression properly.

**Method:** In the study 7 common housekeeping genes in breast cancer tissues were selected and their stability was examined in order to normalize expression of Urokinase Plasminogen Activator (UPA) which is important in metastasis. Reference genes were analyzed with Real-time PCR are as follow: HPRT1, GAPDH, RPLP0, β actin, TFRC, β2M and GUSB. RNA from Breast cancer tissue along with their normal adjacent tissues was extracted using RNX-plus (Cinnagen, Iran). cDNA synthesis was done with reverse transcription kit (Primer design Ltd, UK). Primers and probes were design using GeneRunner version 3.05 and primer Express software version 3. Real-time PCR was carried out using precision 2X mastermix (Primer design Ltd, UK) and fluorescent detection was performed using Applied Biosystems 7500 System. The data was analyzed using geNorm soft ware which uses pairwise comparison approach in order to find the most stable genes.

**Result:** The most stable genes were RPLP0 and HPRT1 while GAPDH was the least stable gene.

**Conclusion:** In this study HPRT1 and RPLP0 were the best house-keeping genes for UPA normalization in breast cancer. Different studies suggest other genes as two of them will be explained. Mc Neill et al, suggest MRPL19 and PPIA as the most stable and RPLP0 as the least stable gene, but Lyng et al, recommend TBP, RPLP0 and PUM1 for normalization. As different studies have special condition and they use some of housekeeping genes in their studies, various genes may be found as the best reference for normalization, some of them are common in various researches. Testing more housekeeping genes will help to find the best genes, but different treatment and situation in research may change the expression of housekeeping and it is better to check the stability of controls based on experiment design to find the proper genes.

**187** Poster

**Correlation between CpG methylation profile of RASSF 1A and RAR2b genes with estrogen receptor (ER) and HER2/neu status in primary breast cancer (BC)**

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**Background:** ER positive BCs are considered prognostically more favorable than ER negative, whereas HER 2/neu positive BCs are associated with worse prognosis. We examined the methylation status in the CpG islands of two major breast tumor-related genes RASSF1A and RAR2b in relation to ER and HER2/neu status in primary BCs.

**Materials and Methods:** Patients with BC (n = 52), randomly selected, were included. Genomic DNA was extracted from archive formalin-fixed paraffin-embedded tumor tissues. DNA methylation was determined by chemical modification of DNA and subsequent double “hot start” Methylation-Specific PCR (MSP), followed by detection on agarose gel. A polyclonal antibody against HER2/neu was used for immunohistochemistry. Results were classified according to the Herceptest criteria: (negative (0/1+), weakly positive (2+) and positive (3+).

**Results:** Methylation of at least one of the genes was observed in 36/52 pts. Methylation of RASSF1A gene was observed in 30/52 pts.