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## Heat shock protein gp96 and CD4+ and CD8+ T-lymphocytes expression as prognostic factors in various molecular types of invasive breast carcinoma

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Breast carcinoma shows extensive clinical and molecular heterogeneity. Glycoprotein gp96 is considered a negative prognostic and predictive factor. Controversy exists over the prognostic role of tumor lymphocytic infiltrates. The goal of this study is to illustrate differences in gp96 and CD4+ and CD8+ T-lymphocytes expression among all immunohistochemical groups of breast carcinoma in relation to the clinical course and outcome of the disease. A retrospective observational study was conducted through processing and analysis of 152 female patient tissue samples previously classified by immunohistochemistry. After immunohistochemical processing, the samples were microscopically analyzed and positive cells were manually calculated in the entire biopsy sample for each patient. In the group of patients with triple negative carcinoma, a significantly higher number of CD4 positive cells in patients with no local recurrence were proven, as well as a significant correlation between a smaller number of CD4 positive cells with a lethal outcome. In the group of patients with Luminal B HER2+ carcinoma, a significantly higher proportion of CD8+ cells in patients with local recurrence were demonstrated. The highest glycoprotein gp96 expression was demonstrated in the group of patients with triple negative carcinoma, while the lowest in patients with Luminal A and Luminal B HER2- carcinoma. This study has shown significantly higher gp96 expression and higher extent of tumor lymphocytic infiltrate in more malignant types of breast carcinoma and represents a significant contribution in affirmation of the prognostic role of these variables.

*Key words: gp96, immunohistochemistry, breast cancer, prognosis, T-lymphocytes*

Breast cancer is the most common malignant disease in the female population and one of the leading causes of death in women worldwide. Globally, 1.7 million new cases are uncovered annually in newly diagnosed females [1]. Heat shock proteins (HSPs) are a ubiquitous group of proteins that are essential to cell life. They possess both chaperone and immunomodulatory characteristics. Overexpression of heat shock proteins in malignant disease is associated with reduced survival and a diminished clinical response to therapy [2]. The main representative of the heat shock proteins within the HSP90 group is glycoprotein gp96 or grp94 (glucose related protein). Gp96 may be stimulated by macrophages as a result of the secretion of various cytokines, which activate dendritic cells and initiate the Th1 specific immunologic response in which then activates cytotoxic T-lymphocytes. Gp96 binds peptides, which are presented to antigen presenting cells (APC) within the major histocompatibility complex (MHC). A translocated gp96 glycopro-

tein (from the endoplasmic reticulum on cell membranes) binds to HER-2 in breast carcinoma cells stabilizing them, thus promoting its further signalization and dimerization [3]. Overexpression of this protein is associated with greater malignancy of tumor cells, greater carcinoma invasiveness, recidivation and metastasis in patients with breast carcinoma [4]. Gp96 expression is higher in cases of tumor recurrence and in patients receiving chemotherapy and may possibly present a central mechanism, which tumor cells use to create an aggressive phenotype and resistance to chemotherapeutic drugs [5]. Gp96 is an attractive target therapy molecule due to its complex immunomodulatory function. Studies are still in the experimental phase. Gp96 vaccines, which use autologous gp96 from tumor tissues, are currently in the clinical trial phase. Specific monoclonal antibodies with targeted action on surface gp96 possess strong antitumor activity.

The application of HSP90 inhibitors in clinical practice is still in the research phase [6, 7]. In the immune response

against tumor cells, T-lymphocytes possess a more prominent role in comparison to humoral immunity, which is mediated by B-lymphocytes. Helper T-lymphocytes (Th) are CD4+ lymphocytes that are activated once an antigen is exposed to the complex of MHC class II molecules, located on the cell surfaces of antigen presenting cells (APC). Cytotoxic T-lymphocytes (CTL) target and destroy tumor cells [8]. There is a greater degree of lymphocytic infiltration in breast carcinoma than in benign tumors, which may be explained by a higher number of antigens found on malignant cells, such as p53, BRCA1 and BRCA2. These antigens are able to elicit local activation and the proliferation of tumor-infiltrative lymphocytes. In overall immune infiltrate, T-lymphocytes are predominantly present [9–13]. The histopathological evaluation of tumor-infiltrating lymphocytes in breast carcinoma may assist in identifying women who may benefit from the immunotherapy [14–16]. The clinical significance of the presence of tumor lymphocyte infiltrates in breast carcinoma is still controversial. Some studies have shown that a dense lymphocytic infiltrates are positive predictive factors in response to neoadjuvant and adjuvant chemotherapy and immunotherapy [17–20]. Recent studies regarding the correlation of tumor lymphocyte infiltrates in breast carcinoma in their relation to the course of disease progression are inconclusive [20–25]. It has been proven that there is a positive association with a greater tumor lymphocytic infiltrate and a HER-2/neu amplification [26]. A sparse tumor lymphocytic infiltrate in breast carcinomas with positive estrogen receptors may be explained by patients in higher age groups and well-differentiated tumors with a lower mitotic index. In triple negative carcinomas, there is a pronounced tumor lymphocytic infiltrate [21, 27]. It has also been shown that there is an association between tumor lymphocytic infiltrate and the stage of the disease, which may be explained by the formation of vascular endothelial growth factor (VEGF) and fibroblastic growth factor (FGF) [28, 29].

Hormone positive and hormone negative tumors display different clinical and pathologic behaviors and thus they possess different interactions within the immune system [30–33].

The aim of this study is to show the differences in glycoprotein gp96 expression and CD4+ and CD8+ T-lymphocyte infiltration in all five groups of breast carcinoma based on immunohistochemistry classification and to establish the relationships that these differences possess in both clinical duration and outcome of the disease.

## Patients and methods

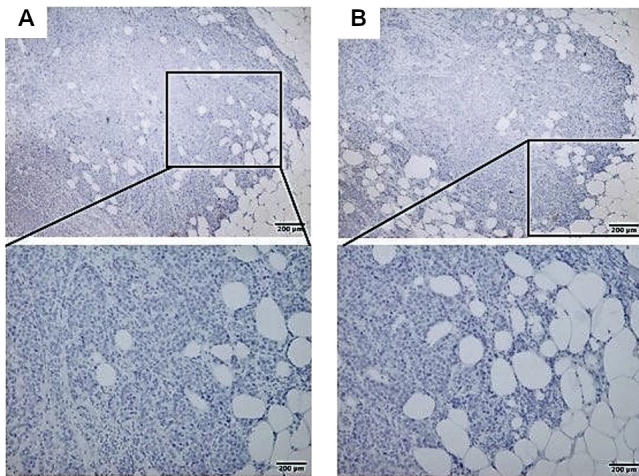
**Patients and clinicopathological data.** Tissue samples of 152 female patients (age range 30–94) operated at the University Hospital Rijeka (2012–2017) were taken for processing and analysis. For each one of the four carcinoma groups 30 biopsies were conducted, in a group of triple negative

carcinoma we obtained 32 biopsies. The patients' samples were divided into five groups according to their previously immunophenotyped biopsies (St. Gallen 2017 classification): Luminal A, Luminal B HER-2 negative, Luminal B HER-2 positive, HER-2 positive and triple negative group. The inclusion criteria were patients diagnosed with invasive breast carcinoma and the availability of a sufficient amount of the representative tumor tissue and clinical data. Clinicopathological data were obtained from patient medical records archived at the University Hospital Rijeka. The retrospective study was approved by the Ethics Committee of the University Hospital Rijeka.

**TMA (tissue microarray) construction and immunohistochemistry.** Processing of the biopsy samples was initiated by making tissue microarrays (TMA) – cylinders of tissue were taken from paraffin blocks containing original (donor) samples and then a new paraffin block was created by embedding 40–88 cylinders into an empty recipient paraffin block. After the marking of all donor blocks, the technical procedure of TMA making was conducted using an Alphelys (Plaisir, France) hand device. Serial 4 µm sections were cut from TMA blocks for immunohistochemical staining. These were mounted on adhesive glass slides (Super Frost Plus), left to dry in the oven at 37°C overnight, deparaffinized and rehydrated.

The following antibodies were used for the immunohistochemical staining: CD4 mouse monoclonal antibody (clone 4B12 Novocastra Leica Biosystems Newcastle Ltd, Newcastle, UK), CD8 mouse monoclonal antibody (clone C8/144B Dako Cytomation, Glostrup, Denmark) and gp96 mouse monoclonal antibody (clone 816803 R&D Systems, Minneapolis, USA). Primary antibodies were diluted using a DAKO antibody diluent according to manufacturer's protocol (CD4 1:75, CD8 1:100, gp96 1:100). For ascertaining protein expression levels, immunohistochemical method Envision was applied together with a Real envision detection system using a Dako Cytomation, Autostainer plus (Glostrup, Denmark) according to the instruction manual from the manufacturer. After immunohistochemical processing, the samples were microscopically analyzed (Olympus BX41 Microscope, CeLL A image processing system) and positive cells (moderate/strong and strong staining intensity) were manually calculated in the entire biopsy sample (three cuts) for each patient (magnification 40x). We have made negative (breast tissue) and positive (pleural mesothelioma tissue) staining controls. Isotype controls were: CD4-IgG1 kappa, CD8-IgG1 kappa, gp96- IgG2 (Figure 1).

**Statistics.** Acquired results were then compared with data concerning the clinical course and outcome of the disease taken from the hospital digital database – age of the female patient, positive axillary lymph nodes at the beginning of treatment, the appearance of local recurrences and distant metastases as well as death rates. Statistical programs Statistica 13.0 (StatSoftInc, Tulsa, USA) and MedCalc (MedCalc Inc., Mariakerke, Belgium) were used for statistical data



**Figure 1.** Isotype control staining. A) GP96, isotype IgG2 (magnification 40 $\times$ ), marked area at magnification 100 $\times$  below; B) CD4, isotype IgG1 kappa (magnification 40 $\times$ ), marked area at magnification 100 $\times$  below.

processing. The results were interpreted on a statistical significance level of  $p=0.05$ . Descriptive statistical methods were used for describing the basic characteristics of the observed groups. Based on the results of the Kolmogorov-Smirnov test, corresponding central tendency measures and variability measures were chosen for quantity variables. Qualitative data are represented using frequencies/percentages. For a comparative statistical analysis of normally distributed variables one way ANOVA analysis was used, together with the Tukey test in post-hoc analysis. For variables that are not normally distributed, comparative analysis was conducted using a Kruskal-Wallis test with multiple comparisons ranking in post-hoc analysis. Comparative statistical analysis of qualitative data was accomplished by using the Chi-square test or the proportion difference test. In order to establish a correlation between variables, we calculated Spearman's rank correlation coefficient.

## Results

**Patients and clinical data.** The study included 152 women with an average age of 66.08 years with standard deviation of 13.72 years. Patients with triple negative carcinomas are statistically significantly younger than the patients with Luminal B HER2 $-$  carcinomas (Tukey HSD test;  $p=0.0015$ ). In 22 (14.47%) patients we established the appearance of local relapse – most patients were in groups with triple negative carcinomas (8.31%), and the minority in Luminal A group (2.7%). 47 patients (30.92%) had distant metastases, which was statistically lower compared with the group of patients without metastases (105; 69.08%,  $\chi^2$  test,  $p=0.001$ ). At the time of diagnosis 98 patients (64.47%) had positive axillary lymph nodes. In total, 59 (38.92%) patients died.

**Immune infiltration.** Post-hoc analysis showed that the extent of immune infiltration is significantly higher in triple

negative type of carcinoma ( $697.94\pm 291.64$ ) and HER2+ carcinoma ( $661.23\pm 293.7$ ) in comparison to Luminal A ( $373\pm 17.54$ ), Luminal B HER2 $-$  ( $372.6\pm 37.39$ ) and Luminal B HER2+ (median 394.5; interquartile range 116, multiple comparison test;  $p<0.03$ ) type of carcinoma.

**Correlation between variables.** Within LUMINAL A carcinoma group (Table S1), we demonstrated weak, but statistically significant positive correlation between gp96 positive cells/overall number of tumor cells and gp96/CD4 positive cells ratio, also between distant metastases and positive axillary lymph nodes.

We demonstrated statistically significant, positive and weak, correlation between CD8 positive cells/overall immune infiltrate ratio and relapses, distant metastases and positive axillary lymph nodes. We demonstrated also a positive and weak statistically significant correlation between death and distant metastases. Between CD8 positive cells/overall immune cells ratio and gp96 positive cells/CD8 positive cells ratio, we demonstrated statistically significant, weak and negative, correlation. A moderate positive statistically significant correlation was found between gp96 positive cells/overall tumor cells ratio and gp96 positive cells/CD8 positive cells ratio. A moderate negative statistically significant correlation was found between gp96 positive cells/CD4 positive cells ratio and CD4 positive cells/overall immune infiltrate ratio.

Within LUMINAL B HER2 $-$  carcinoma group (Table S2), we demonstrated a positive and weak correlation between gp96 positive cells/overall tumor cells ratio and gp96 positive cells/CD8 positive cells ratio, between distant metastases and positive axillary lymph nodes and also between distant metastases and death. A moderate positive statistically significant correlation was found between gp96 positive cells/CD4 positive cells ratio and gp96 positive cells/CD8 positive cells ratio. A good and negative correlation was found between gp96 positive cells/CD4 positive cells ratio and CD4 positive cells/overall immune infiltrate ratio and also between gp96 positive cells/CD8 positive cells ratio and CD8 positive cells/overall immune infiltrate ratio.

Within LUMINAL B HER2+ carcinoma group (Table S3), we demonstrated a positive and weak correlation between gp96 positive cells/overall tumor cells ratio and gp96 positive cells/CD8 positive cells ratio and also between CD8 positive cells/overall immune infiltrate ratio and relapses. A good and positive correlation was found between gp96 positive cells/overall tumor cells ratio and gp96 positive cells/CD4 positive cells ratio, gp96 positive cells/CD4 positive cells ratio and gp96 positive cells/CD8 positive cells ratio and also between death and distant metastases. We demonstrated an excellent negative correlation between gp96 positive cells/CD8 positive cells ratio and CD8 positive cells/overall immune infiltrate ratio.

Within HER2+ carcinoma group (Table S4) we demonstrated a weak positive correlation between gp96 positive cells/overall tumor cells ratio, gp96 positive cells/CD4



positive cells ratio and gp96 positive cells/CD8 positive cells ratio, between CD8 positive cells/overall immune infiltrate ratio and positive axillary lymph nodes and also between distant metastases and positive axillary lymph nodes. A good positive correlation was found between distant metastases and death. A good negative correlation was found between gp96 positive cells/CD8 positive cells ratio and CD8 positive cells/overall immune infiltrate ratio.

Within TRIPLE NEGATIVE carcinoma group (Table S5), we demonstrated a weak positive correlation between CD8 positive cells/overall immune infiltrate ratio and relapses, CD8 positive cells/overall immune infiltrate and metastases, metastases and positive axillary lymph nodes, positive axillary lymph nodes and death. A weak negative correlation was found between gp96 positive cells/CD8 positive cells ratio and CD8 positive cells/overall immune infiltrate ratio. A moderate positive correlation was found between gp96 positive cells/overall tumor cells ratio and gp96 positive cells/CD4 positive cells ratio and also between gp96 positive cells/CD4 positive cells ratio and gp96 positive cells/CD8 positive cells ratio. A moderate negative correlation was found between gp96 positive cells/CD4 positive cells ratio and CD4 positive cells/overall immune infiltrate ratio. A good positive correlation was found between gp96 positive cells/overall tumor cells ratio and gp96 positive cells/CD8 positive cells ratio. An excellent positive correlation was found between distant metastases and death.

**Age-related data.** ANOVA test was performed which showed statistically significant difference within age according to the classification of carcinoma (Table S6 ANOVA;  $p=0.005$ ).

Post-hoc analysis pointed out that patients with triple negative carcinoma are statistically significantly younger than the patients with Luminal B HER2- carcinoma (Table S7, Tukey HSD test;  $p=0.0015$ ). Statistically significant age-related difference between other groups of patients was not found (Table S7; Tukey HSD test;  $p>0.055$ ).

According to linear regression analysis results, there is a statistically significant correlation of CD4 positive cells and age in LUMINAL A and LUMINAL B HER 2+ types of carcinoma (Table S8). The number of CD4 positive cells decreases with age in LUMINAL A type of carcinoma (negative correlation, moderate connectivity), while in LUMINAL B HER2+ type of carcinoma the number of CD4 positive cells is increasing with age.

In the LUMINAL A and HER2+ types of carcinoma, there is a statistically significant correlation of ratio of CD4 positive cells/overall immune infiltration and age of patients according to linear regression analysis (Table S9). In LUMINAL A type of carcinoma number of CD4 positive cells is decreasing with age (negative correlation, moderate connection) and in HER2+ type of carcinoma, portion is increasing with age (positive correlation, moderate connection).

Linear regression analysis shows that there is a statistically significant correlation between CD8 positive cells and age in

LUMINAL A and LUMINAL B HER2+ types of carcinoma (Table S10). The number of CD8 positive cells decreases with age in LUMINAL A type (negative correlation moderate connection), and increases in LUMINAL B HER2+ carcinoma type (positive correlation, moderate connection).

Linear regression analysis results show a statistically significant correlation of ratio of CD8 positive cells/overall immune infiltration and age of patients only in LUMINAL A type of carcinoma (Table S11), in which ratio of CD8 positive cells decreases with age (negative correlation, moderate connection).

In HER2+ type of carcinoma we established small, but statistically significant positive correlation between expression of GP96 and age (Table S12;  $r=0.38$ ;  $p=0.04$ ). For other types of carcinoma such significance is not established (Table S12;  $p>0.3$ ).

**CD4+ cells.** Post-hoc analysis showed that the number of CD4 positive cells is statistically significantly higher in triple negative ( $218.97\pm 132.55$ ) and HER2+ type of carcinoma ( $166.00\pm 128.33$ ) (Figure 2, Figure 3). The number of CD4 positive cells decreases with age in Luminal A type of carcinoma, while in Luminal B HER2+ type of carcinoma the number of CD4 positive cells is increasing. In patients with triple negative type of carcinoma, the number of CD4 positive cells is statistically significantly higher in cases when there is no local relapse (Mann-Whitney test;  $p=0.033$ ). It has been shown that in the cases of lethal outcomes, the number of CD4 positive cells was statistically significantly lower (Mann-Whitney test;  $p=0.013$ ).

**CD4+ cells in overall immune infiltration.** In Luminal A type of carcinoma, the number of CD4 positive cells is decreasing with age and in HER2+ type of carcinoma is increasing with age. In patients with triple negative carcinoma, the share of CD4 positive cells is statistically significantly higher in cases when there are no distant metastases. In a group of patients without lethal outcomes, there is a statistically significantly higher portion of CD4 positive cells in triple negative type of carcinoma (Mann-Whitney test;  $p=0.0009$ ).

**CD8+ cells.** Post-hoc analysis shows that the number of CD8 positive cells is statistically significantly higher in triple negative carcinoma type ( $334.78\pm 132.80$ ) and HER2+ type ( $311.90\pm 182.80$ ), Tukey HSD test;  $p<0.001$ , (Figure 4, Figure 5). The number of CD8 positive cells decreases with age in Luminal A type and increases in Luminal B HER2+ carcinoma type.

**CD8+ cells in overall immune infiltration.** Linear regression analysis results show a statistically significant correlation of share of CD8 positive cells in immune infiltration and age of patients only in carcinoma of Luminal A type in which share of CD8 positive cells decreases with age. In cases of Luminal B HER2+ carcinoma, a statistically significantly higher share of CD8 positive cells was in the group with local relapse (Mann-Whitney test;  $p=0.049$ ). In triple negative type of carcinoma, a share of CD8 positive cells is

statistically significantly higher in a group of patients with the lethal outcomes (Mann-Whitney test;  $p=0.049$ ).

**Gp96 expression.** Post-hoc analysis showed the highest number of gp96 positive cells in triple negative carcinoma type ( $1295\pm635$ ), and the lowest in Luminal A ( $471\pm288$ ) and Luminal B HER2- ( $672\pm299$ ) type of carcinoma (Tukey HSD test;  $p<0.001$ ) (Figure 6, Figure 7). In HER2+ type of carcinoma, we established a statistically significant positive correlation between the expression of gp96 and age. It has been shown that gp96 expression is statistically significantly higher in cases of HER2+ carcinomas in patients with local relapse (Mann-Whitney test;  $p=0.035$ ).

**Gp96 expression and immune infiltration.** Post-hoc analysis showed that the ratios of gp96 positive cells and immune infiltration are statistically significantly higher in triple negative ( $2.22\pm1.53$ ) and Luminal B HER2+ ( $2.2\pm1.84$ ) type of carcinoma in comparison to Luminal A type of carcinoma ( $1.25\pm0.75$ ; Tukey HSD test;  $p<0.03$ ).

**Gp96/CD4+ cells.** In a group of Luminal A type, we established a positive correlation with the age of patients. In a group HER2+, we established a negative correlation with the age of patients. In triple negative group, the ratio of expression was significantly higher in patients with positive axillary lymph nodes at the time of diagnosis and in those with lethal outcomes. We showed that gp96 and CD4 positive cells ratio in triple negative type of carcinoma is statistically significantly higher in patients with positive axillary lymph nodes at the time of diagnosis in comparison to those without (Mann-Whitney;  $p=0.0019$ ). Statis-

tically significantly higher ratio of gp96 and CD4 positive cells was seen in triple negative carcinoma type in patients with lethal outcomes in comparison to those without lethal outcomes (Mann-Whitney;  $p=0.041$ ).

**Gp96/CD8+ cells.** By analyzing the expression of gp96 and CD8+ T-lymphocytes ratios, we did not establish a significant difference in the ratio among groups, significant correlation to age, positive axillary lymph nodes at the time of diagnosis, appearance of local relapse and distant metastases.

**Gp96 positive cells and overall tumor cells.** Post-hoc analysis showed the highest gp96 expression in triple negative carcinoma type ( $53.74\pm25.52$ ) % and the lowest in Luminal A ( $20.82\pm12.51$ )% and Luminal B HER2- ( $28.77\pm12.76$ )% type of carcinoma (Tukey HSD test;  $p<0.001$ ). In HER2+ type of carcinoma, we established a statistically significant positive correlation between gp96 expression and age. It has been shown that gp96 expression is statistically significantly higher in cases of HER2+ carcinoma in patients with local relapse (Mann-Whitney test;  $p=0.032$ ).

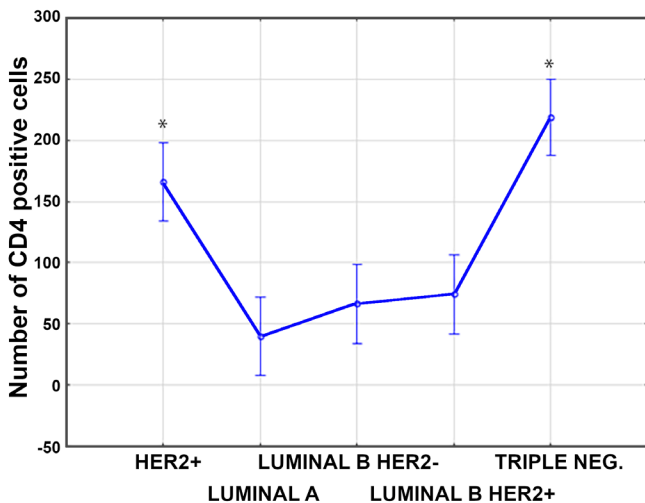


Figure 2. Comparison of arithmetic means of CD4 positive cells for different types of breast carcinoma. Pointers represent 95% reliability interval. Post-hoc analysis showed that the number of CD4 positive cells is statistically significantly higher (\*,  $p<0.001$ ) in triple negative ( $218.97\pm132.55$ ) and HER2+ group of carcinoma ( $166.00\pm128.33$ ) in comparison to LUMINAL A ( $40.10\pm27.36$ ), LUMINAL B HER2- ( $66.27\pm40.03$ ) and LUMINAL B HER2+ ( $74.07\pm49.50$ ) group of carcinoma (Tukey HSD test;  $p<0.001$ ).

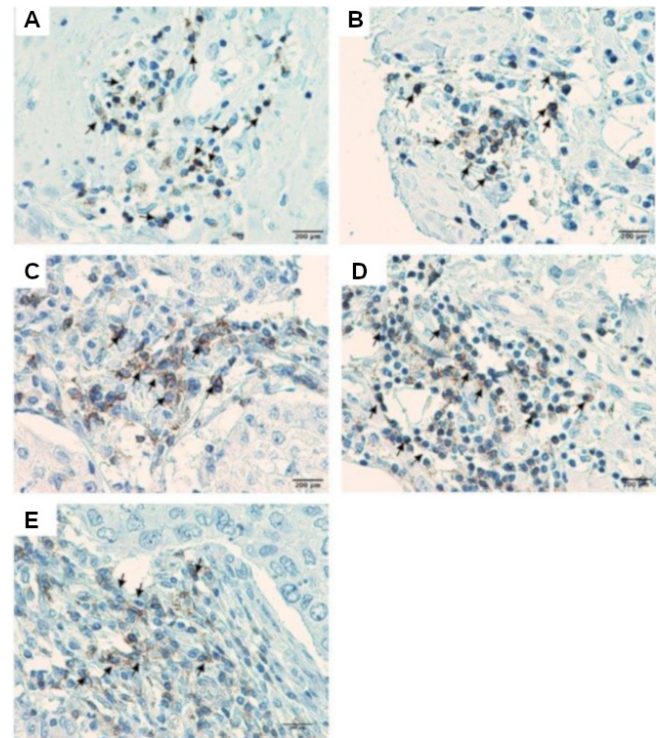


Figure 3. Immunohistochemical staining using CD4 mouse monoclonal antibody (clone 4B12 Novocastra Leica Biosystems Newcastle Ltd, Newcastle, UK): stromal and peritumoral localization of lymphocytic infiltrate. Moderate/strong cytoplasmic staining (Olympus BX41 Microscope, CeLL A, magnification 40 $\times$ ). Arrow markers are used to point out the stained cells. A) Luminal A carcinoma, B) Luminal B HER2- carcinoma, C) Luminal B HER2+ carcinoma, D) HER2+ carcinoma, E) TRIPLE NEGATIVE carcinoma.

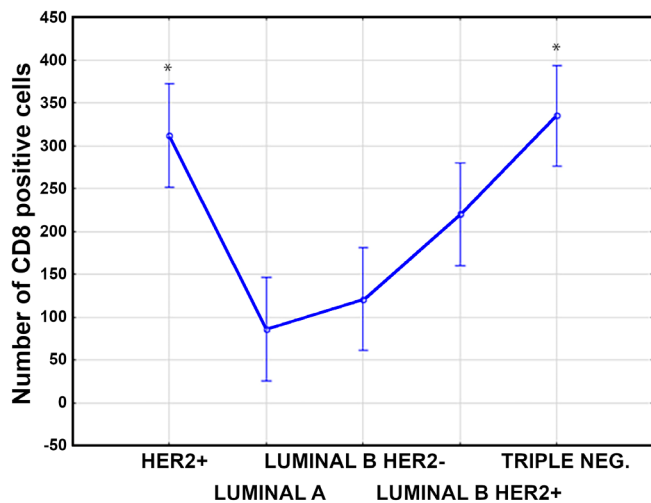


Figure 4. Comparison of arithmetic means of CD8 positive cells for different types of breast carcinoma. Pointers represent 95% reliability interval. Post-hoc analysis shows that the number of CD8 positive cells is statically significantly higher (\*,  $p < 0.001$ ) in triple negative group of carcinoma (334.78+132.80) and HER2+ (311.90+182.80) group of carcinoma in comparison to LUMINAL A (86.16+56.96) and LUMINAL B HER2- group of carcinoma (121.10+69.21) (Tukey HSD test;  $p < 0.001$ ). Number of CD8 positive cells is statistically significantly higher in LUMINAL B HER2- (219.97+206.58) group of carcinoma in comparison to number of CD8 cells in LUMINAL A group of carcinoma (Tukey HSD test;  $p = 0.02$ ).

## Discussion

Breast cancer is a very heterogeneous malignant disease with different biological behavior between and within different immunohistochemical groups. In our research, in the total immune infiltrate, the highest numbers of cells were T- lymphocytes, with fewer B- lymphocytes, plasma cells, NK cells and macrophages. Immunological infiltrate had stromal, peritumoral and intratumoral localization, corresponding to the data from literature [10, 11, 16]. Triple negative and HER-2 positive carcinomas had the highest density of immune infiltrations, as proved by Loi et al. They also demonstrated that a dense tumor lymphocytic infiltrate in triple negative carcinomas was a favorable prognostic factor [20]. In this study, the correlation between the size of the total immune infiltrate with the age of the patient, the positive axillary lymph nodes at the time of diagnosis, the occurrence of local recurrence and distant metastases and the death was not found, among of any type of cancer. Tsang et al. reported in 2014 the correlation of the density of lymphocytic infiltrates with high histological grade, the presence of necrosis and the absence of fibrotic zones in carcinoma. There was no evidence of correlation of lymphocyte infiltration density with the age of the patient, lymph node involvement, and the presence of an extensive intraductal component in the carcinomas [22]. Helal et al. in 2013 demonstrated the correlation of lymphocyte infiltrate (T-lymphocytes) density with

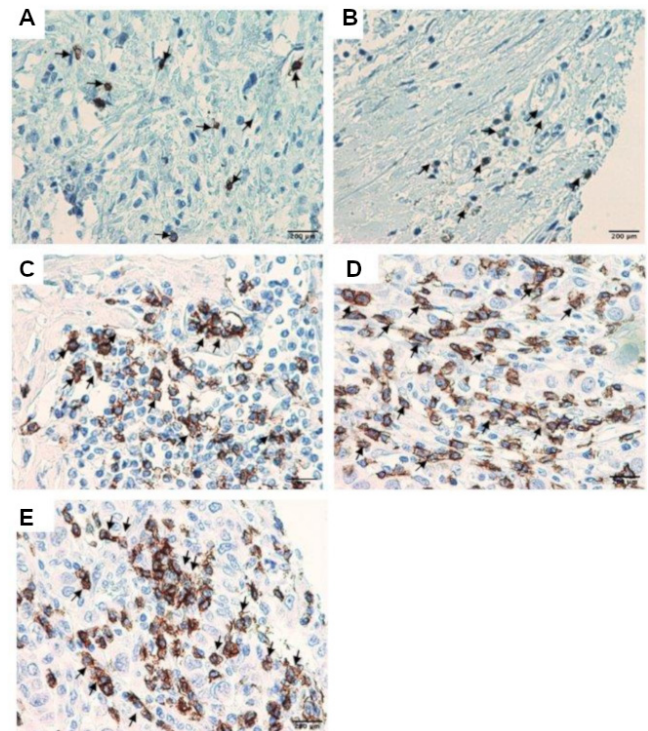


Figure 5. Immunohistochemical staining using CD8 mouse monoclonal antibody (clone C8/144B Dako Cytomation, Glostrup, Denmark): stromal, intratumoral and peritumoral localization of lymphocytic infiltrate, strong cytoplasmic staining (Olympus BX41 Microscope, CeLL A, magnification 40 $\times$ ). Arrow markers are used to point out the stained cells. A) Luminal A carcinoma, B) Luminal B HER2- carcinoma, C) Luminal B HER2+ carcinoma, D) HER2+ carcinoma, E) TRIPLE NEGATIVE carcinoma.

the age of the patients, higher stage of disease (III and IV) and poorer cell differentiation [9]. The association of tumor lymphocytic infiltrate density and HER-2/neu amplification was demonstrated, which can be explained by the activation of NF kappa B signal pathway, which initiates lymphocytic activation and inflammatory response after HER-2 signaling [26]. Calabro et al. in 2009 proved that denser lymphocytic infiltration in hormone-positive carcinomas was associated with shorter and in hormone-negative ones with longer survival rates [30].

Mohammed et al. in 2012 demonstrated the association of the density of immune infiltrate with higher histological grade and vascular invasion and the absence of estrogen and progesterone receptors. They also demonstrated the association of lymphocyte infiltration density with longer total survival in ductal carcinoma patients [31]. In the group of patients with triple negative carcinomas, the proportion of CD4+ T-lymphocyte share is higher in patients with no registered metastases or lethal outcomes. Macchetti et al. in 2006 published a study that demonstrated a correlation of CD4+ T-lymphocyte infiltrate in early breast cancer patients (T1-T2) with positive axillary lymph nodes, while the corre-



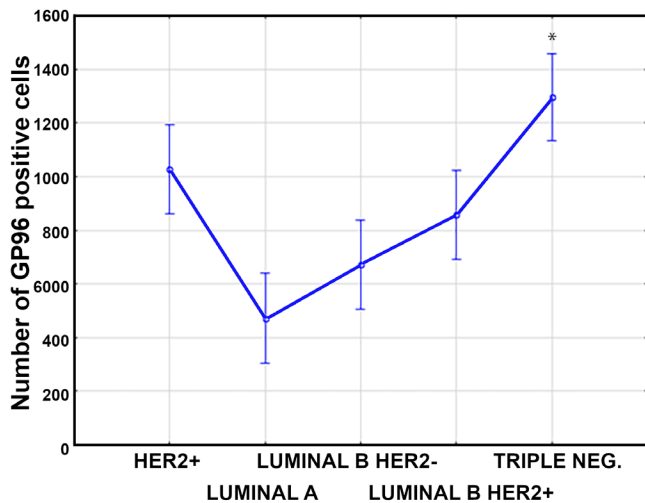


Figure 6. Comparison of arithmetic means of the number of positive GP96 cells in different types of breast carcinoma. Pointers represent 95% reliability interval. Post-hoc analysis showed the highest number of GP96 cells in triple negative group of carcinoma (1295 + 635) and the lowest in LUMINAL A (471+288) and LUMINAL B HER2- (672+299) (Tukey HSD test;  $p < 0.001$ ) group of carcinoma. Number of positive GP96 cells in triple negative carcinoma is statistically significantly higher (\*,  $p = 0.002$ ) in comparison to LUMINAL B HER2- group of carcinoma (857+475) (Tukey HSD test;  $p = 0.002$ ), and there is no statistically significantly different from the number of GP96 cells in HER2+ (1295+635) (Tukey HSD test;  $p = 0.155$ ) group of carcinoma.

lation with CD8+ T-lymphocyte infiltrate was not statistically significant [21]. In the analysis of the CD8+ T-lymphocyte share in the total immune infiltrate, we showed a significantly higher proportion in triple negative carcinomas compared to Luminal A and Luminal B HER2- group and a significantly lower proportion in Luminal A carcinomas compared to Luminal B HER2+ and HER2+ group. In the group of patients with Luminal B HER2+ carcinoma, a significantly higher proportion of CD8+ cells in patients with local recurrence was demonstrated. In the group of patients with triple negative carcinoma, a significantly higher proportion of CD8+ cells was demonstrated in the cases of lethal outcomes. In comparison with our results, Mahmoud et al. in 2011 published that tumor infiltration of CD8+ lymphocytes in breast carcinoma was associated with longer survival rates [32]. Ali et al. in 2014 have proven that tumor infiltration with cytotoxic T-lymphocytes in breast cancer with negative estrogen receptors was associated with reduced relative death risk. In breast cancer positive to estrogen receptors, the association of immune infiltration and longer survival was not proven [33]. Rathore et al. in 2013 demonstrated the association of CD3+ lymphocyte infiltrate density with positive axillary lymph nodes [23]. In 2008, La Rocca et al. demonstrated a greater number of CD4+ and CD8+ T-lymphocytes in ductal carcinoma of patients without metastases in axillary lymph nodes [24].

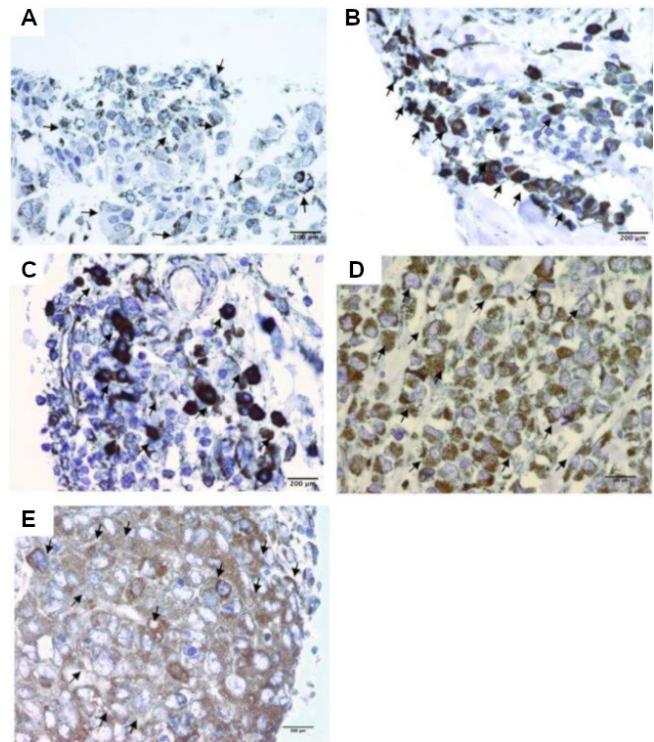


Figure 7. Immunohistochemical staining using Gp96 mouse monoclonal antibody (clone 816803 R&D Systems, Minneapolis, USA): strong membranous and cytoplasmic staining (Olympus BX41 Microscope, CeLL A, magnification 40 $\times$ ). Arrow markers are used to point out the stained cells. A) Luminal A carcinoma, B) Luminal B HER2- carcinoma, C) Luminal B HER2+ carcinoma, D) HER2+ carcinoma, E) TRIPLE NEGATIVE carcinoma.

In 2009, Matkowski et al. demonstrated that tumor infiltration with CD4+ and CD8+ lymphocytes was associated with lymph node involvement and poor prognosis of the disease. However, there was no correlation between tumor immune infiltration with tumor size, histological grade, hormone receptor expression and HER-2 expression [25]. The highest glycoprotein gp96 expression was demonstrated in the group of patients with triple negative carcinoma, the lowest in patients with Luminal A and Luminal B HER2- carcinoma. No study has been published so far regarding all molecular types of breast carcinoma and gp96 expression. In the group of patients with HER2+ carcinoma, a significant positive correlation between gp96 expression and the age of the patients was demonstrated, as well as a significant association between gp96 expression and local recurrence. In our research we have proven greater gp96 expression in more malignant molecular types of breast carcinoma, which is in agreement with published data [3-5].

In conclusion, the interaction of tumor metabolism and immune response of oncological patients is a very complex process. This study has shown a significantly higher gp96 expression and a higher extent of tumor lymphocytic infiltrate in more malignant types of breast carcinoma. Further



(prospective) research on glycoprotein gp96 expression and tumor lymphocytic infiltration is required for a more precise definition of the correlation between these two variables and its significance in the prognostic context of different immunohistochemical groups of breast carcinoma. We consider this paper a great contribution in affirmation of the prognostic role of the gp96 expression and tumor lymphocytic infiltration. Therefore, we hope that these results will contribute to a better understanding of clinical and pathological characteristics of carcinomas with various immunophenotypes and development of the new treatment methods in the future.

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## Heat shock protein gp96 and CD4+ and CD8+ T-lymphocytes expression as prognostic factors in various molecular types of invasive breast carcinoma

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### Supplementary Information

**Table 1. Spearman's correlation coefficients-Luminal A carcinoma group. Statistically significant results are highlighted in red color.**

LUMINAL A CARCINOMA	GP96 vs TC	GP96 vs CD4	GP96 vs CD8	CD4 vs IMI	CD8 vs IMI	Relapse	Metastasis	Pos.ax.l.n.	Death
GP96 vs TC	1.00								
GP96 vs CD4	<b>0.42</b>	1.00							
GP96 vs CD8	<b>0.63</b>	<b>0.51</b>	1.00						
CD4 vs IMI	0.15	<b>-0.63</b>	-0.26	1.00					
CD8 vs IMI	0.13	-0.20	<b>-0.46</b>	0.31	1.00				
Relapse	0.00	-0.05	-0.14	0.11	<b>0.47</b>	1.00			
Metastasis	0.00	-0.15	-0.19	0.23	<b>0.43</b>	0.29	1.00		
Pos.ax.l.n.	0.00	0.02	-0.13	-0.03	<b>0.38</b>	0.04	<b>0.45</b>	1.00	
Death	0.09	0.15	0.02	-0.11	0.24	0.14	<b>0.43</b>	<b>0.39</b>	1.00

TC - tumor cells; IMI - immune infiltrate; Pos.ax.l.n.- positive axillary lymph nodes.

**Table 2. Spearman's correlation coefficients-Luminal B HER2- carcinoma. Statistically significant results are highlighted in red color.**

LUMINAL B HER2- CARCINOMA	GP96 vs TC	GP96 vs CD4	GP96 vs CD8	CD4 vs IMI	CD8 vs IMI	Relapse	Metastasis	Pos.ax.l.n.	Death
GP96 vs TC	1.00								
GP96 vs CD4	<b>0.20</b>	1.00							
GP96 vs CD8	<b>0.45</b>	<b>0.50</b>	1.00						
CD4 vs IMI	0.38	<b>-0.61</b>	-0.06	1.00					
CD8 vs IMI	0.24	-0.38	<b>-0.66</b>	0.34	1.00				
Relapse	-0.37	0.28	-0.34	-0.37	<b>-0.11</b>	1.00			
Metastasis	0.28	0.01	0.03	0.16	<b>0.15</b>	-0.22	1.00		
Pos.ax.l.n.	-0.22	-0.07	-0.21	0.00	<b>0.04</b>	0.16	<b>0.34</b>	1.00	
Death	0.17	0.17	0.08	0.01	0.11	-0.04	<b>0.44</b>	<b>0.39</b>	1.00

TC - tumor cells; IMI - immune infiltrate; Pos.ax.l.n.- positive axillary lymph nodes.

**Table 3. Spearman's correlation coefficients-Luminal B HER2+ carcinoma. Statistically significant results are highlighted in red color.**

LUMINAL B HER2+ CARCINOMA	GP96 vs TC	GP96 vs CD4	GP96 vs CD8	CD4 vs IMI	CD8 vs IMI	Relapse	Metastasis	Pos.ax.l.n.	Death
GP96 vs TC	1.00								
GP96 vs CD4	<b>0.62</b>	1.00							
GP96 vs CD8	<b>0.36</b>	<b>0.70</b>	1.00						
CD4 vs IMI	0.07	<b>-0.53</b>	-0.42	1.00					
CD8 vs IMI	0.14	-0.31	<b>-0.81</b>	0.42	1.00				
Relapse	-0.12	-0.16	-0.34	0.11	<b>0.37</b>	1.00			
Metastasis	-0.16	0.00	-0.18	-0.13	<b>0.22</b>	0.51	1.00		
Pos.ax.l.n.	0.07	0.05	-0.04	-0.17	<b>0.12</b>	-0.11	<b>0.18</b>	1.00	
Death	-0.03	0.16	-0.04	-0.06	0.16	0.40	<b>0.66</b>	<b>0.10</b>	1.00

TC - tumor cells; IMI - immune infiltrate; Pos.ax.l.n.- positive axillary lymph nodes.



**Table 4. Spearman's correlation coefficients-HER2+ carcinoma. Statistically significant results are highlighted in red color.**

HER2+	GP96 vs TC	GP96 vs CD4	GP96 vs CD8	CD4 vs IMI	CD8 vs IMI	Relapse	Metastasis	Pos.ax.l.n.	Death
GP96 vs TC	1.00								
GP96 vs CD4	<b>0.01</b>	1.00							
GP96 vs CD8	<b>0.34</b>	<b>0.39</b>	1.00						
CD4 vs IMI	0.42	<b>-0.78</b>	0.07	1.00					
CD8 vs IMI	0.11	0.07	<b>-0.74</b>	-0.25	1.00				
Relapse	0.40	-0.17	0.24	0.34	<b>-0.06</b>	1.00			
Metastasis	0.14	0.06	-0.10	0.00	<b>0.22</b>	-0.02	1.00		
Pos.ax.l.n.	0.18	0.11	-0.13	0.02	<b>0.29</b>	0.20	<b>0.30</b>	1.00	
Death	0.29	0.18	0.03	-0.07	0.30	0.05	<b>0.64</b>	<b>0.19</b>	1.00

TC - tumor cells; IMI - immune infiltrate; Pos.ax.l.n.- positive axillary lymph nodes.

**Table 5. Spearman's correlation coefficients-TRIPLE NEGATIVE carcinoma. Statistically significant results are highlighted in red color.**

TRIPLE NEGATIVE	GP96 vs TC	GP96 vs CD4	GP96 vs CD8	CD4 vs IMI	CD8 vs IMI	Relapse	Metastasis	Pos.ax.l.n.	Death
GP96 vs TC	1.00								
GP96 vs CD4	<b>0.56</b>	1.00							
GP96 vs CD8	<b>0.77</b>	<b>0.52</b>	1.00						
CD4 vs IMI	0.12	<b>-0.52</b>	0.39	1.00					
CD8 vs IMI	-0.11	0.44	<b>-0.45</b>	-0.96	1.00				
Relapse	-0.08	0.18	-0.02	-0.32	<b>0.32</b>	1.00			
Metastasis	0.08	0.25	-0.04	-0.40	<b>0.38</b>	0.36	1.00		
Pos.ax.l.n.	0.16	0.42	0.22	-0.12	<b>0.12</b>	0.11	<b>0.37</b>	1.00	
Death	0.08	0.37	-0.05	-0.46	0.45	0.33	<b>0.81</b>	<b>0.28</b>	1.00

TC - tumor cells; IMI - immune infiltrate; Pos.ax.l.n.- positive axillary lymph nodes.

**Table 6. Descriptive statistics of age (mean, standard deviation, minimally and maximum value) of patients divided by the molecular classification of carcinoma.**

Carcinoma classification	Number of patients	Age				
		Arithmetic mean	Standard deviation	Min	Max	p-value*
LUMINAL A	30	64.00	14.20	32	81	
LUMINAL B HER2-	30	73.20	11.34	48	89	
LUMINAL B HER2+	30	67.77	14.67	38	94	<b>0.005</b>
HER2+	30	65.30	11.04	47	85	
TRIPLE NEG.	32	60.50	14.31	30	83	
Total	152	66.08	13.72	30	94	

**Table 7. Post- hoc analysis Tukey HSD test between patient's age according to types of carcinoma.**

	LUMINAL A	LUMINAL B HER2-	LUMINAL B HER2+	HER2+	TRIPLE NEG.
LUMINAL A		0.055	0.805	0.996	0.836
LUMINAL B HER2-	0.055		0.503	0.140	<b>0.002</b>
LUMINAL B HER2+	0.805	0.503		0.951	0.194
HER2+	0.140	0.951	0.609		0.996
TRIPLE NEG.	0.836	<b>0.002</b>	0.194	0.609	

**Table 8. Number of CD4 positive cells in correlation to age: results of linear regression analysis. Statistically significant results are highlighted in red.**

	<b>r</b>	<b>p-value</b>
LUMINAL A	<b>-0.37</b>	<b>0.04</b>
LUMINAL B HER2-	-0.11	0.55
LUMINAL B HER2+	<b>0.40</b>	<b>0.03</b>
HER2+	0.28	0.14
TRIPLE NEG.	-0.23	0.19

r- Pearson's coefficient correlation; p- level of significance.

**Table 9. Ratio of CD4 cells/overall immune infiltration depending on age.**

	<b>r</b>	<b>p-value</b>
LUMINAL A	<b>-0.38</b>	<b>0.004</b>
LUMINAL B HER2-	-0.11	0.55
LUMINAL B HER2+	0.34	0.063
HER2+	<b>0.42</b>	<b>0.021</b>
TRIPLE NEG.	-0.26	0.16

**Table 10. Expression of CD8 in correlation to age: results of linear regression analysis. Statistically significant results are highlighted in red.**

	<b>r</b>	<b>p-value</b>
LUMINAL A	<b>-0.37</b>	<b>0.04</b>
LUMINAL B HER2-	0.09	0.65
LUMINAL B HER2+	<b>0.37</b>	<b>0.05</b>
HER2+	-0.14	0.47
TRIPLE NEG.	-0.05	0.78

r- pearson's coefficient correlation; p- level of significance.

**Table 11. Ratio of CD8 cells in overall immune infiltration in dependence of age.**

	<b>r</b>	<b>p-value</b>
LUMINAL A	<b>-0.38</b>	<b>0.04</b>
LUMINAL B HER2-	-0.06	0.75
LUMINAL B HER2+	0.23	0.22
HER2+	-0.03	0.89
TRIPLE NEG.	0.19	0.31

**Table 12. Expression of GP96 according to age: results of linear regression analysis. Statistically significant results are shown in red.**

	<b>r</b>	<b>p-value</b>
LUMINAL A	-0.07	0.70
LUMINAL B HER2-	-0.04	0.83
LUMINAL B HER2+	0.13	0.48
HER2+	<b>0.38</b>	<b>0.04</b>
TRIPLE NEG.	0.16	0.37

r- pearson's coefficient correlation; p -level of significance.