# PROGNOSTIC SIGNIFICANCE OF *P21* PROTEIN IN BREAST CANCER

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## ABSTRACT

*Breast cancer is the most common malignancy in women. Bearing in mind these circumstances, a review of new molecular mechanisms underlying breast cancer progression, resistance and various aspects of existing therapeutic methods would lead to new insights for biologists and clinicians. In this regard, we conducted a study covering recent advances in breast cancer biology with a focus on the p21 protein. The study included 147 patients diagnosed with invasive breast cancer. The presence of non-invasive lesions was noted in each invasive breast cancer and surrounding tissue. p21 expression was determined by reading the percentage of nuclear expression in epithelial cells of invasive breast cancer and non-invasive lesions. Results showed that expression of p21 increases with the progression of cytological changes in the epithelium; it is significantly higher in invasive breast cancer compared to non-invasive lesions ($p<0.001$). There is a difference in p21 expression between different molecular subtypes of breast cancer ($p=0.004$). Statistically significantly higher values of p21 expression were observed in those breast cancers that showed overexpression of HER2 compared to HER2-negative tumors ($p=0.001$). Depending on Ki67 expression, the highest p21 expression is in the group with high Ki67 expression values ($p=0.019$). The increase in p21 expression in tumor cells was accompanied by a statistically significantly reduced expression of ER ($p=0.015$, $\rho=-0.225$) and PR ($p=0.027$, $\rho=-0.205$). p21 protein plays an important role in proliferation, malignant transformation, as well as in progression from non-invasive lesions to invasive breast cancer.*

***Keywords***: *Cyclin-Dependent Kinase Inhibitor p21, breast cancer, molecular subtype, immunohistochemistry.*

## INTRODUCTION

Breast cancer (*IBC*) is the most common malignancy (30% of all new tumor cases) and the second leading cause of tumor death in women (15% of all tumor deaths) (1) and is an important public health problem worldwide. In 2020, there were 2.3 million cases of illness and 685.000 deaths (2). In the Republic of Serbia, about 4.000 newly discovered cases of this disease are registered every year, which represents more than a quarter of all malignant diseases in women, with most European countries showing a decreasing trend in mortality from breast cancer, while Serbia, Poland and Romania have recorded less in the last period as well favorable trends.Thus, in 2017, the highest mortality rates were recorded in Serbia (20.6/100.000 women) (3).

In the past two decades, new therapies, such as immunotherapy and targeted therapies have emerged as a result of the discovery of new molecules and signaling pathways involved in breast cancer proliferation. However, a significant proportion of patients remain resistant to therapy or relapse, indicating an incomplete understanding of cancer metabolism (4). Bearing in mind these circumstances, a review of new molecular mechanisms underlying breast cancer progression, resistance and various aspects of existing therapeutic methods would lead to new insights for biologists and clinicians. In this regard, we conducted a study covering recent advances in breast cancer biology with a focus on the *p21* protein.

*p21*, a 21 *KDa* protein encoded by the *CDKN1A* gene, is a member of the *Cip/Kip* family of *CDKIs* in addition to *p27* and *p57*. *p21* is the first discovered transcriptional target of the *p53* protein. It is capable of inactivating all *CDKs,* thereby inhibiting cell cycle progression (5). It inhibits the kinase activity of the cyclin-*CDK* complex by interacting with cyclins via two cyclin binding motifs (*Cy1* and *Cy2*). This leads to inhibition of phosphorylation of the *pRb* family of proteins and subsequent association with *E2F* and formation of the *DREAM* complex, leading to cell cycle arrest (6, 7). *p21* plays a dual role in cell cycle progression depending on its expression level (8). High levels of *p21* inhibit the kinase activity of the cyclin-*CDK* complex leading to inhibition of cell cycle progression, while low levels act as a factor in the formation of the cyclin *D/CDK4,6* complex and promote its activation leading to cell cycle progression (9). Despite its role in arresting cell proliferation and its ability to promote differentiation and cell senescence, recent studies suggest that under certain conditions *p21* can promote cell proliferation and oncogenicity (10). Accordingly, *p21* is often misregulated in human cancers, but its expression, depending on the cellular context and circumstances, suggests that it may act as a tumor suppressor or as an oncogene (10).These conflicting observations have undoubtedly increased the importance of *p2*1 in the field of tumor biology. Moreover, to date, no consensus has been reached on the relationship between *p21* and clinicopathological parameters, and the characteristics of *p21* expression and its clinical/prognostic significance in human breast cancer remain unclear.

Since the data of previous researches were different, and also there is no consensus and clear guidelines regarding the use of *p21* expression in routine clinical diagnosis of breast cancer, we conducted this study to analyze the expression of *p21* in *IBC*, but also in non-invasive breast lesions (*NIL*) with the aim of determining whether *p21* is a useful adjunctive tool for distinguishing between *NIL* and *IBC*.

Furthermore, we examined the possible correlation of this marker with the clinicopathological characteristics of *IBC* as a possible link with its progression.

## MATERIAL AND METHODS

### Study Design

The study was conducted in accordance with the Declaration of Helsinki, approved by the decision of the Ethics Committee number 01/17/2290, included 147 patients with a diagnosis of breast cancer, who were diagnosed and treated at the University Clinical Center Kragujevac, Serbia in the period from 2012 to 2017.

Pathohistological analysis of tumors on *H&E* stained specimens was performed on the operative material obtained by tumerectomy, quadrantectomy and/or mastectomy with dissection of regional lymph nodes (11).

The presence of non-invasive lesions *(NIL)* [*in situ* lobular and ductal carcinoma *(ISC)*, lobular and ductal atypical hyperplasia (*AH*) and normal ductal and acinar epithelium (*NE*)] was noted in each *IBC* and surrounding tissue. Based on microscopic analysis, *IBCs* were classified into three groups: ductal, lobular, and a group of other histological types. E-cadherin expression differentiated ductal from lobular breast cancer. At the same time, all relevant macroscopic, pathohistological and prognostic parameters (tumor size, histological type and grade, nodal status, presence of necrosis, intra and peritumoral mononuclear infiltrate, perineural, lymphatic and vascular invasion, molecular subtype IBC and disease stage) were defined (12).

*IBCs* are classified according to the recommendations into four molecular groups: *Luminal A, Luminal B, HER2 +* and *TNBC* (13).

### Immunohistochemical (*IHC*) procedure

Tissue sections were fixed in 10% Neutral Buffered and stabilized formalin solution, pH 7.0 and embedded into paraffin. *IHC* was performed on a tissue section of a representative paraffin block of each patient. Tissue sections 4 μm thick were applied to adherent slides (*SuperFrost® Plus,* VWR, Leuven, Belgium), then deparaffinized in xylene and rehydrated in decreasing alcohol concentrations. After epitope retrieval, endogenous peroxidase was blocked with 3% hydrogen peroxide for 5 minutes. The preparations were incubated

with primary monoclonal and polyclonal antibodies at room temperature for the recommended duration. The following antibodies were used, ready for use or in appropriate dilution: *p21$^{WAF1/CIP1}$* (SX118, 1:50, M7202, DAKO, Denmark), mAb *ER* (1D5, RTU, IR657, DAKO, Denmark), mAb *PR* (PgR636, RTU, IR068, DAKO, Denmark), pAb *HER2* (1:1200, AO485, DAKO, Denmark), *Ki67* (1:200, MIB-1, IR626, DAKO, Denmark). After washing the primary antibody, tissue sections were incubated with commercial biotinized secondary antibody at room temperature, for the recommended duration (*En Vision* FLEX HRP, RTU, K8000). The *IHC* reaction was visualized using 3,3'-diaminobenzidine tetrahydrochloride (*DAB*). The preparations were finally contrasted with Mayer's hematoxylin (Hematoxylin M, HEMM-OT-1L, Biognost, Croatia), and the coverslips were mounted with Canada balsam. Tissue samples in which incubation with the primary antibody was omitted were used as negative controls of the *IHC* reaction, and *IBCs* with known expression of the analyzed markers were used as positive controls. Slides were analyzed at 100x, 200x, and 400x magnifications, using a light microscope (*AxioScop 40*, Carl Zeiss, Germany). Representative sections were photographed using a digital camera (*AxioCam ICc1*, Carl Zeiss, Germany).

**Evaluation of *IHC* Staining**

Immunohistochemical stains were rated by two independent pathologists, who were blinded to clinical follow-up data at the time of analysis. In cases of differently assessed expression, agreement was reached by joint analysis of the preparation and consultation with a third pathologist.

Analysis of estrogene (*ER*) and progesterone receptors (*PR*) expression was performed using Allred score (14) as the sum of the percentage of positive nuclei of tumor cells and the intensity of *IHC* staining. The Allred score ranges from 0 to 8.

The expression of human epidermal growth factor receptor 2 (*HER2*) was analyzed based on standard recommendations (15). Depending on the continuity and intensity of membrane staining, all *IBCs* were classified into *HER2* negative (0 and 1+) and *HER2* positive (3+). Equivocal *HER2* (2+) was retested by silver in situ hybridization (*SISH*) technique after which patients were classified as *HER2* positive or negative *IBCs*.

*Ki67 IHC* expression was defined as the percentage of positive tumor cells per 100 counted in the zone of highest tumor proliferation. According to the previously defined limit value of *Ki67* expression in our laboratory, *IBCs* are classified into 3 groups: low (*Ki67* <15%), medium (*Ki67*: 15-30%) and high proliferative activity (*Ki67*> 30%) (16, 17).

*p21* expression was determined as the percentage of nuclear expression in *IBC* and *NIL* epithelial cells. By analyzing the expression, we defined the *cut off* value for *p21*. Based on the obtained result, we divided all *IBCs* into *p21* positive and *p21* negative

**Statistical data processing**

The commercial software package *SPSS* (version 22.0, *SRSS Inc., Chicago, IL*) was used for statistical processing of the data obtained. In the analysis of the obtained results we used: descriptive statistics methods, *Man-Whitney* test, *Kruskal-Wallis* test, $\chi 2$ test, *Pearson* or *Spearman* correlation coefficient, *ROC* curve (with determination of cut off value, sensitivity and specificity). By determining the sensitivity and specificity of the test, the level of practical reliability of statistical analysis was determined. All reported p values were 2-sided and $p < 0.05$ was considered statistically significant.

## RESULTS

The experimental research group included 147 women diagnosed with IBC, average age 58, with the youngest patient being 29 and the oldest 84. In 79 patients, in situ carcinoma (ISC) was present simultaneously with IBC, in 82 atypical hyperplasia (AH), and in 109 cases, fields of glandular parenchyma without signs of epithelial proliferation and atypia were noted (NE - normal epithelium of ducts and acini). The average size of the cancer was 22.5 mm (the smallest 9 mm and the largest 68 mm).The average expression of estrogene receptors is 54.27±35.48%, while the average expression of progesterone receptors is 34.49±34.43. Presented through the Allred score, the average value of the score for estrogens was 5.36±3.17 and for progesterone 3.92±3.21. Other clinicopathological characteristics of IBC are shown in Table 1.

**Table 1.** Clinicopathological characteristics of breast cancer

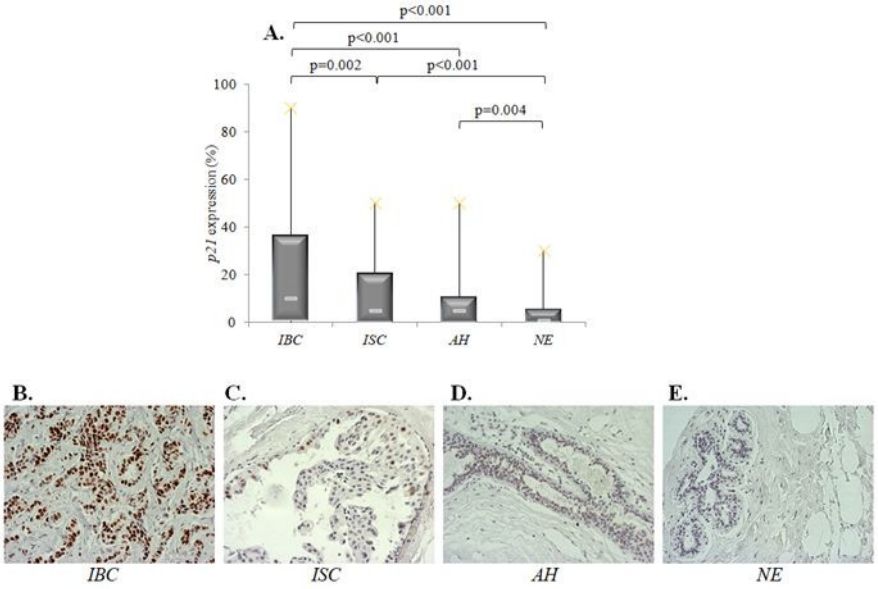
| *Variables* | | *N* | *%* |
|---|---|---|---|
| Side | left | 66 | 44.9 |
| | right | 81 | 55.1 |
| Histological type | lobular | 18 | 12.4 |
| | ductal | 123 | 84.8 |
| | other | 4 | 2.8 |
| Histological grade | HG1 | 17 | 11.9 |

| *Variables* | | *N* | *%* |
|---|---|---|---|
| | HG2 | 73 | 51 |
| | HG3 | 53 | 37.1 |
| Nuclear grade | NG1 | 17 | 15.2 |
| | NG2 | 64 | 57.1 |
| | NG3 | 31 | 27.7 |
| Mitotic index | grade 1 | 19 | 42.2 |
| | grade 2 | 20 | 44.4 |
| | grade 3 | 6 | 13.3 |
| Tumor necrosis | absent | 26 | 21.7 |
| | present | 94 | 78.3 |
| Desmoplasia | low | 17 | 16.3 |
| | medium | 55 | 52.9 |
| | high | 32 | 30.8 |
| Periductal elastosis | low | 19 | 20.0 |
| | medium | 20 | 44.4 |
| | high | 16 | 35.6 |
| Perineural invasion | absent | 101 | 68.7 |
| | present | 46 | 31.3 |
| Lymphatic invasion | absent | 72 | 48.9 |
| | present | 75 | 51.1 |
| Vascular invasion | absent | 113 | 76.9 |
| | present | 34 | 23.1 |
| HER2 | negative | 115 | 79.3 |
| | positive | 30 | 20.7 |
| Ki67 | low | 30 | 20..9 |
| | medium | 42 | 29.4 |
| | high | 71 | 49.7 |
| Molecular subtypes | Lum A | 30 | 20.4 |
| | Lum B | 76 | 51.7 |
| | HER2 + | 19 | 12.9 |
| | TNBC | 22 | 15 |
| *T* status | *T1* | 48 | 35.8 |
| | *T2* | 64 | 47.8 |
| | *T3* | 9 | 6.7 |
| | *T4* | 13 | 9.7 |
| *N* status | *N0* | 50 | 37.3 |
| | *N1* | 48 | 35.8 |
| | *N2* | 19 | 14.2 |
| | *N3* | 17 | 12.7 |

**Expression of *p21* in relation to cytological changes in epithelium**

The average value of *p21* expression was calculated, and a statistically significant increase was observed with increasing invasiveness (in *NE* 2%, in *AH* 7%, in *ISC* 11% and in *IBC* 23%) (*Kruskal-Wallis, p<0.001*). A statistically significant difference was absent only in the case of the comparison of the *ISC* and *AH* groups (*Mann-Whitney U, p=0.06*), while this difference was shown between all other groups (Figure 1A).Immunohistochemical expression of *p21* in different histo- and cytomorphological changes is shown in Figure 1B-E.

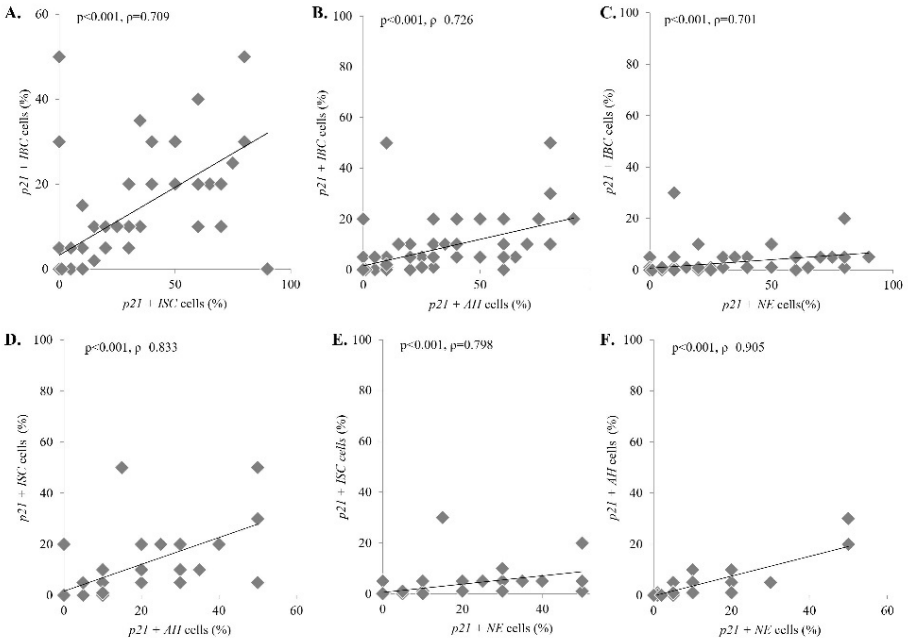
**Figure 1.** Expression of p21 in relation to cytological changes in the epithelium. A.




A statistically significant difference was observed between each of the mentioned groups except between the *ISC* and *AH* groups (*Mann Whitney U, p=0.06*). The result is shown as the median. Microscopic image of *p21* expression in various histo and cytomorphological changes: **B.** *IBC*. **C**. *ISC*. **D.** *AH*. **E.** *NE* (immunohistochemical analysis, original magnification 200x).

A strong positive correlation was found between *p21* expression in all mentioned changes (*Spearman ρ*). With the increase in *p21* expression in *IBC* cells, the expression in *NIL* cells also increases (Figure 2). Also, the increase in p21 expression in *ISCs* was accompanied by an increase in *AH* and *NE*. With increasing expression in *AH*, expression in *NE* also increases.

**Figure 2.** Correlation of p21 expression between groups in relation to cytological changes in the epithelium (Spearman ρ).

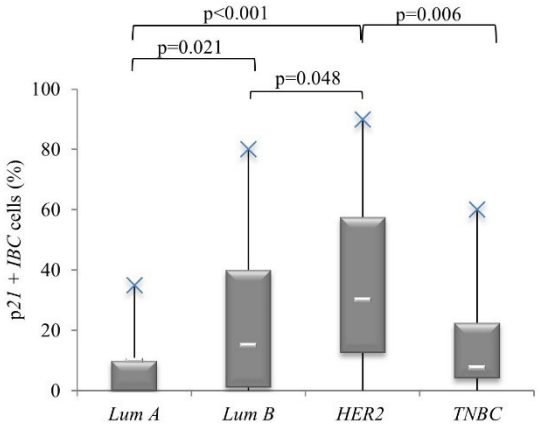



A strong positive correlation was found between all groups: **A**. *IBC* and *ISC* (*p<0.001, ρ=0.709*), **B**. *IBC* and *AH* (*p<0.001, ρ=0.726*), **C**. *IBC* and *NE* (*p<0.001, ρ=0.701*), **D**. *ISC* and *AH* (*p<0.001, ρ=0.833*), **E**. *ISC* and *NE* (*p<0.001, ρ=0.798*) and **F**. *AH* and *NE* (*p<0.001, ρ=0.905*).

**Expression of *p21* in tumor cells in relation to the molecular subtype of *IBC***

When looking at the expression of *p21* in relation to the molecular subtypes of *IBC*, a statistically significant difference was observed among the different subtypes (*Kruskal-Wallis, p=0.004*). The highest expression was observed in the *HER2+* subtype of *IBC*. In relation to the *HER2+* subtype of *IBC*, molecular subtypes *Lum B* and *Lum A* had a statistically significantly lower value of *p21* expression, while the lowest value was recorded in *TNBC* (Figure 3).

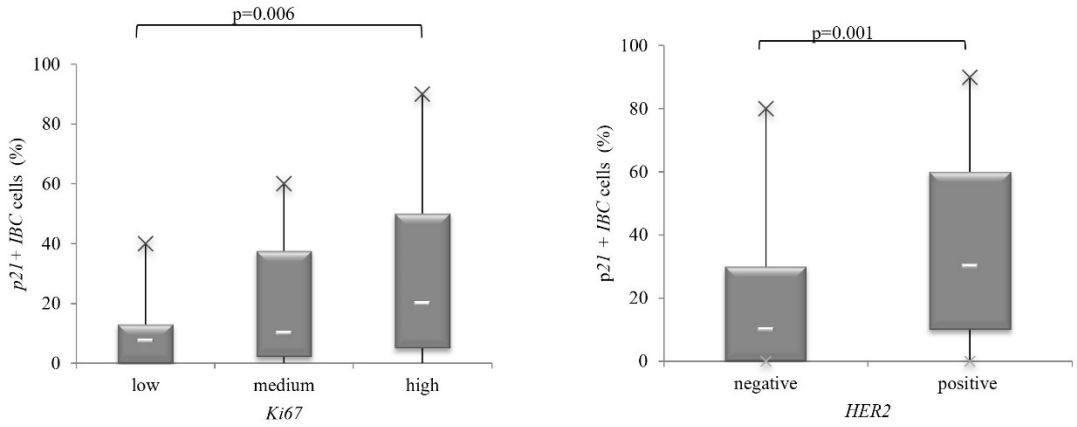
**Figure 3.** Expression of p21 in different molecular subtypes of IBC.




A statistically significant difference was shown between the following groups: *Lum A* and *Lum B* (*Mann-Whitney U, p=0.021*), *Lum A* and *HER2* (*Mann-Whitney U, p<0.00*), *Lum B* and *HER2* (*Mann-Whitney U, p=0.048*), *HER2* and *TNBC* (*Mann-Whitney U, p=0.006*). The result is presented as median.

Statistically significantly higher values of *p21* expression were observed in those *IBCs* that showed overexpression of *HER2* compared to *HER2*-negative tumors. Depending on *Ki67* expression, the highest *p21* expression is in the group with high *Ki67* expression values (Figure 4).

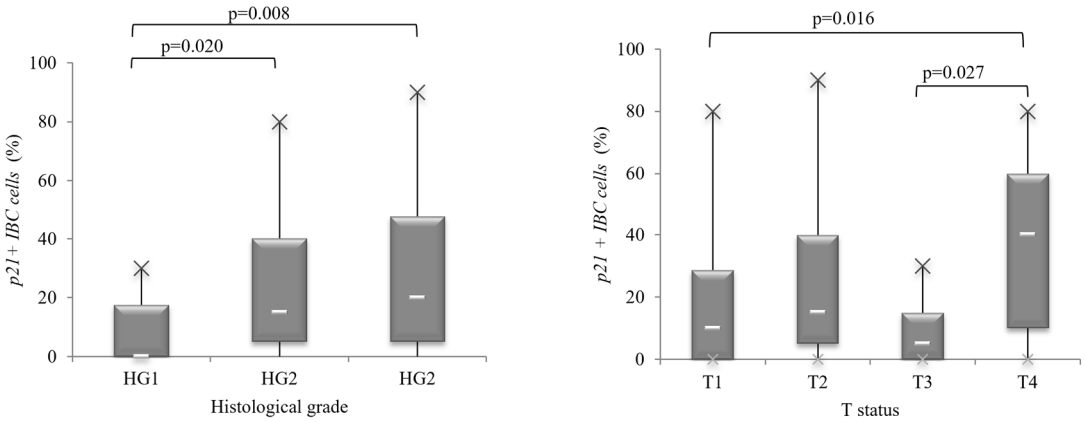
**Figure 4.** p21 expression depending on Ki67 expression and HER2 expression.




**A**. *p21* expression in tumor cells depends on *Ki67* expression. The result is shown as the median (*Kruskal-Wallis, p=0.019*). **B**. There is a significant difference in *p21* expression depending on *HER2* expression. The result is shown as the median (*Mann Whitney U, p=0.001*).

In relation to the nuclear grade, a statistically significantly higher expression of *p21* was shown in *IBCs* of nuclear grade 3 compared to nuclear grades 1 and 2. Also, *IBCs* belonging to the *T4* group, in relation to the *T* status, had a significantly higher expression in relation toto groups *T1* and *T2* (Figure 5).

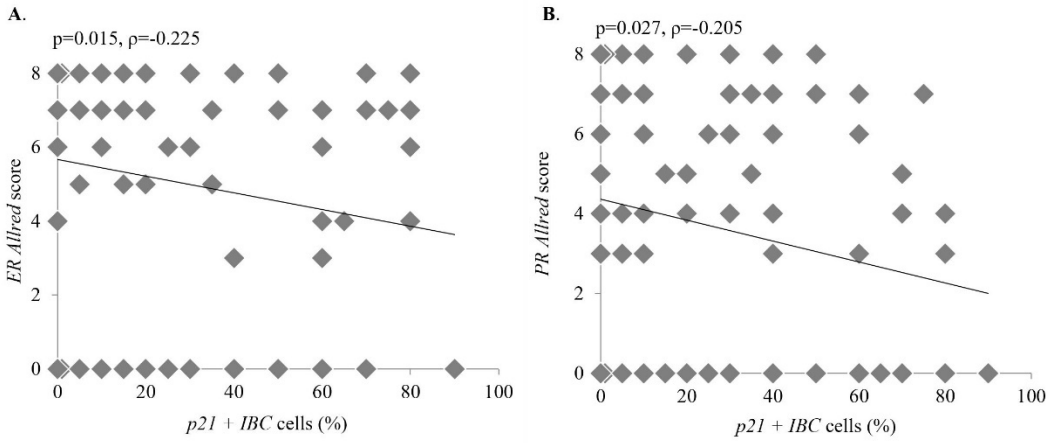
**Figure 5.** Expression of p21 depending on expression of nuclear grade and T status.




Expression of *p21* in tumor cells depends on **A**. nuclear grade (*Kruskal-Wallis, p=0.026*) and **B**. *T* status (*Kruskal-Wallis, p=0.05*). The result is shown as the median.

When the expression of *p21* was observed in relation to the expression of receptors for estrogens and progesterone, it was observed that with the increase in the expression of *p21* in *IBC*, the expression of receptors for estrogens and progesterone decreases significantly (*Spearman ρ*) (Figure 6).

**Figure 6.** Expression of *p21* depending on the expression of *ER* and *PR*.

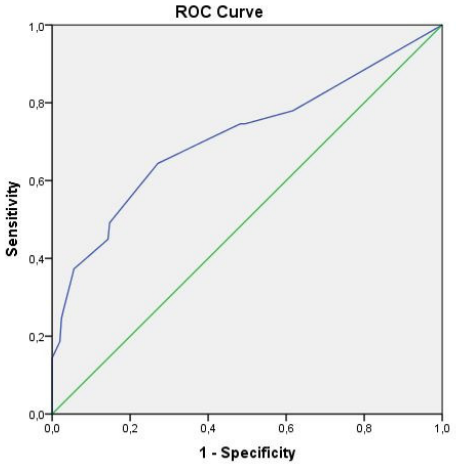



The expression of *ER* and *PR* was analyzed through the *Allred* score. The increase in *p21* expression in tumor cells was accompanied by a statistically significantly reduced expression of **A**. *ER (p=0.015, ρ=-0.225)* and **B**. *PR (p=0.027, ρ=-0.205)* (small negative correlation).

**Expression of *p21* as a marker of breast cancer progression**

By analyzing the expression of *p21*, it was shown that its increase can be a marker of breast cancer progression, with an expression value of 7.5% as a treshold value, with a sensitivity of 64.4% and a specificity of 72.9% (*AUC=0.712, p<0.001*) (Figure 7).

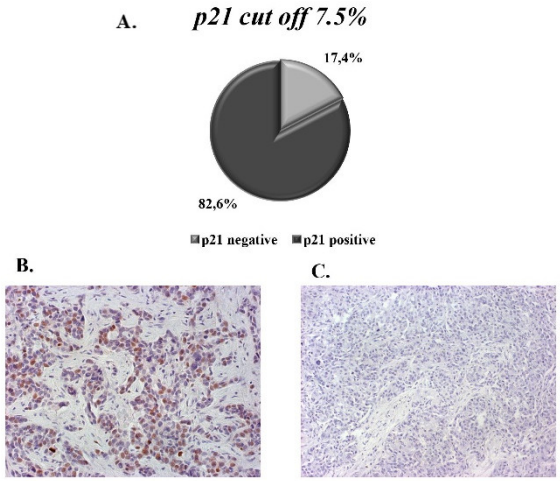
**Figure 7.** *ROC* curve of *p21* expression in *NIL* and *IBC*.




The calculated value of *AUC=0.712* with a sensitivity of 64.4% and a specificity of 64.4% determined a threshold value of 7.5%.

Using the obtained threshold value, all *IBCs* were further divided into two groups: negative whose *p21* expression value was ≤7.5%, and positive whose *p21* expression was >7.5% (Figure 8A).The immunohistochemical expression of *p21* relative to the threshold value is shown in Figure 8B-C.

**Figure 8. A**. Frequency of *p21+* and *p21- IBC* in relation to the treshold value of *p21* expression.




Microscopic image of *p21* expression in relation to the threshold value: **B**. *p21+* and **C**. *p21-* (immunohistochemical analysis, original magnification 200x).

A statistically significant association with the molecular subtype of *IBC* as well as with *HER2* expression was demonstrated. A significant difference in the percentage of *IBC* molecular subtypes can be observed in the *HER2+* subtype, where 19.7% are *p21* positive, while no *p21* negative *IBC* belongs to the *HER2+* molecular subtype. Also, when looking at *HER2* status, it was found that all *IBCs* that were negative in relation to *p21* expression were also *HER2* negative, while 33.8% of *p21* positive ones were *HER2* positive. Other clinicopathological characteristics did not show a statistically significant association (Table 2).

**Table 2.** Association between *p21* expression in *IBC* and examined clinicopathological characteristics.

| *Variables* | | *p21 cut off 7.5%* | | *Chi-Square* | *p* |
|---|---|---|---|---|---|
| | | - | + | | |
| Mononuclear infiltrate | absent | 1 (9.1%) | 3 (5.8%) | 0.666 | 0.881 |
| | low | 4 (36.4%) | 20 (38.5%) | | |
| | medium | 5 (45.5%) | 20 (38.5%) | | |
| | high | 1 (9.1%) | 9 (17.3%) | | |
| Histological type | lobular | 1 (6.7%) | 5 (6.7%) | 0.622 | 0.733 |
| | ductal | 14 (93.3) | 67 (89.3) | | |
| | other | 0 (0.0%) | 3 (4.0%) | | |
| Histological grade | HG1 | 2 (12.5%) | 8 (11.0%) | 0.364 | 0.834 |
| | HG2 | 7 (43.8%) | 38 (52.1%) | | |
| | HG3 | 7 (43.8%) | 27 (37.0) | | |
| Nuclear grade | NG1 | 0 (0.0%) | 6 (9.7%) | 2.096 | 0.351 |
| | NG2 | 7 (53.8%) | 37 (59.7%) | | |
| | NG3 | 6 (46.2%) | 19 (30.6%) | | |
| Tumor necrosis | absent | 2 (15.4%) | 17 (27.0%) | 0.278 | 0.598 |
| | present | 11 (84.6%) | 46 (73.0%) | | |
| Perineural invasion | absent | 13 (81.3%) | 49 (64.5%) | 1.015 | 0.314 |
| | present | 3 (18.8) | 27 (35.5%) | | |
| Lymphatic invasion | absent | 5 (31.3%) | 37 (48.7%) | 0.993 | 0.319 |
| | present | 11 (68.8%) | 39 (51.3%) | | |
| Vascular invasion | absent | 12 (75.0%) | 57 (75.0%) | 0.000 | 1.000 |
| | present | 4 (25.0%) | 19 (25.0%) | | |
| Molecular subtypes | *Lum A* | 1 (6.3%) | 10 (13.2%) | 11.490 | 0.009 |
| | *Lum B* | 8 (50.0%) | 42 (55.3%) | | |
| | *HER2 +* | 0 (0.0%) | 15 (19.7%) | | |
| | *TNBC* | 7 (43.8%) | 9 (11.8%) | | |
| *HER2* | negative | 16 (100.0%) | 49 (66.2%) | 5.895 | 0.015 |
| | positive | 0 (0.0%) | 25 (33.8%) | | |
| *Ki67* | low | 2 (13.3%) | 10 (13.5%) | 1.075 | 0.584 |
| | medium | 6 (40.0%) | 20 (27.0%) | | |
| | high | 7 (46.7%) | 44 (59.5) | | |
| *T* status | *T1* | 3 (20.0%) | 22 (32.4%) | 2.924 | 0.404 |
| | *T2* | 9 (60.0%) | 34 (50.0%) | | |
| | *T3* | 2 (13.3%) | 3 (4.4%) | | |
| | *T4* | 1 (6.7%) | 9 (13.2%) | | |
| *N* status | *N0* | 4 (26.7%) | 23 (33.3%) | 1.504 | 0.681 |
| | *N1* | 8 (53.3%) | 26 (37.7%) | | |
| | *N2* | 1 (6.7%) | 10 (14.5%) | | |
| | *N3* | 2 (13.3%) | 10 (14.5%) | | |

## DISCUSSION

Although extensive research related to breast cancer has been conducted in recent years, there is still a need to understand the basic aspect of tumorigenesis. Cell cycle control proteins are known to play an important role in the pathogenesis of breast cancer (18). Each phase of the cell cycle is strictly regulated in normal cells. However, upon exposure to mitogenic stimuli, these regulatory components become deregulated, which predisposes to cellular transformation of breast epithelial cells. Numerous studies have implicated roles for oncogenic and tumor-suppressive components in various types of human cancers, including breast cancer initiation and development (19-21). *p21* was initially considered a tumor suppressor protein because it was recognized as a major mediator of cell cycle arrest. Conversely, it has been proposed that *p21* may also function as an oncogene because it can inhibit apoptosis. Therefore, *p21* is considered a dual-behavior protein, as its expression can cause potential benefits or harmful effects in breast cancer (22).

We calculated the average value of *p21* expression in different breast lesions and found that the highest expression was in *IBC* and gradually decreased from *ISC*, through *AH* to

*NE*. Identical results were obtained by *Wei et al*., i.e. showed that *p21* protein was highly expressed in cancer samples, compared to normal breast tissue (23). In the same study, they showed that *p21* protein expression is significantly associated with larger tumor diameter, higher grade, and lymph node metastases, which indicates a poor prognosis of the disease. Other authors have also shown that the level of *p21* expression is significantly elevated in patients with breast cancer compared to patients with benign breast lesions (24-28). *Zohny et al* showed that *p21* expression was associated with histological grade 3 tumors and the presence of lymph node metastases, which together indicate that high expression of this marker is associated with advanced breast cancer (25). *Barbareschi* examined the expression of *p21* in changes in the breast and came to the results that show a gradual increase in the expression of this marker from normal epithelium, through well-differentiated and then poorly differentiated *ISC* to *IBC* where the expression was also the highest (26). Furthermore, high expression was accompanied by high histological grade, but there was no correlation with other investigated clinicopathological characteristics of the tumor. If the role of *p21* is only inhibition of the cyclin/CDK complex, which is required for the transition from G1 to S, and inhibition of DNA replication, high expression of p21 could result in reduced cell proliferation. However, in our study, p21 was significantly increased in IBC compared to NIL. We can explain this by the presence of mutated non-functional forms of p21. Balbin et al investigated p21 in 36 primary breast cancers and found a mutation in this molecule due to the replacement of arginine with tryptophan ($p21^{R94W}$) (29). This mutation was attributed to a tumor-specific change, as it was not observed in *DNA* extracted from peripheral blood cells of the same patient. Functional analysis of the $p21^{R94W}$ protein produced in different expression systems revealed that this mutation causes an impairment in the ability of *p21* to inhibit cyclin*A/CDK2*, cyclin*B/CDK1*, cyclin*D/CDK4*, and cyclin*D1/CDK6*. These data suggest that the $p21^{R94W}$ protein may participate in breast carcinogenesis because it cannot inhibit a number of cyclin/*CDK* complexes. Differences in *p21* expression were also observed in changes in other organs. Thus, strong positive nuclear staining with *p21* was observed in full-thickness epithelium in squamous cell carcinoma, while in keratoacanthoma it was limited to the peripheral and suprabasal layers. Both the level and intensity of staining for *p21* were higher in squamous cell carcinoma compared to keratoacanthoma (30).

We showed the association of *p21* expression with high nuclear grade and larger tumor diameter and tumors in *T4* disease status, high *Ki67* proliferative index, positive *HER2* status and negative *ER* and *PR*. Others obtained similar results and showed that overexpression of *p21* is associated with positive nodal status, larger tumor diameter, and worse prognosis in breast cancer patients (31). The association of high *p21* expression with high proliferative activity is particularly interesting in the context of our research. Increased cell proliferation in *p21* positive cases has been previously described (32). High *p21* expression in highly proliferative cells may reflect failed attempts to arrest proliferation. This may result from the presence of other cell cycle regulatory pathways, which bypass the *p21*-mediated cell cycle block, such as c-Myc or B-Myb (33, 34). In addition, overexpression of *CDK2* and cyclin *A* has been reported to reverse the inhibitory effect of high *p21* expression (*35, 36*). Mutation of the retinoblastoma protein (*pRb*) can also lead to increased expression of *p21* via deregulation of the transcription factor *E2F-1 (37, 38)*.

By analyzing the molecular subtypes of breast cancer, we found that *p21* expression was highest in the *HER2* positive subtype of *IBC*. This is further supported by the fact that *p21* expression was highest in *HER2* overexpressing tumors. Others have found that *HER2* overexpression positively correlates with *p21* in breast tumors and that there is a significant correlation of *p21* positivity with worse disease-free survival (39). Studies have shown that in breast cancer cells, *HER2* can contribute to the translocation of *p21* from the nucleus to the cytoplasm, resulting in the loss of its tumor suppressor function (40). There is increasing evidence that the function of *p21* is related to its localization in cells. When localized in the cytoplasm, *p21* functions as an oncogene, thus promoting cell proliferation and progression through the cell cycle, while nuclear localization of *p21* is involved in prodifferentiation and senescence-promoting effects (40, 41).

## CONCLUSION

Our results confirmed that increased expression of *p21* may indicate malignant transformation of breast changes and progression of *IBC*. The treshold value of *p21*-positive tumor cells allows the separation of patients with *NIL* from those with *IBC*, so *IHC* analysis of *p21* expression can be used as an additional diagnostic test in separating benign from malignant changes in the breast. Such observations may recommend this marker for use in diagnostic purposes.

## ETHICS APPROVAL

The study was conducted in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national) and the Helsinki Declaration of 1975, as revised in 2013.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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