MEDICAL UNIVERSITY

“Prof. Dr Paraskev Stoyanov” - Varna

Faculty of Medicine

Deparment of General and Clinical pathology, Forensic medicine and Deontology

Martina Georgieva Stoeva, MD

IMMUNOHISTOCHEMICAL EXPRESSION OF THE NECROPTOSIS MARKER RIPK3 IN BREAST CANCER

THESIS SUMMARY

of a dissertation for award

of educational and scientific degree

"Doctor of Philosophy"

Scientific specialty: „Pathology and Cytopathology“

Supervisor
Prof. Maria Tzaneva, MD, PhD

Varna, 2022

The dissertation contains 133 standard pages and is illustrated with 50 tables and 42 figures. The literature includes 205 literature sources, of which 4 in Cyrillic and 201 in Latin.

The dissertation is discussed and directed to the defense of the Department Council of the Department of General and Clinical Pathology, Forensic Medicine and Deontology at MU "Prof. Dr. Paraskev Stoyanov ”- Varna on January 10, 2022.

The public defense of the dissertation will be held on March 11, 2022, Friday, before a scientific jury composed of:

External members:

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1. Prof. Dr. Veselin Belovezhdov, PhD

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GLOSSARY

СЗО – Световна здравна организация
ФКБ – фибро-кистична болест
BRCA1 – Breast cancer 1
DFS – Disease-free survival
ER – Estrogen receptor
HER2 - Human epidermal growth factor receptor 2
IS – Intensity score
MLKL – Mixed Lineage Kinase Domain Like Pseudokinase
NOS – not otherwise specified
PR – Progesterone receptor
PS – Proportion score
RIPK1 – Receptor-interacting serine/threonine-protein kinase 1
RIPK3 - Receptor Interacting Serine/Threonine Kinase 3 MLKL
TS – Total score
WHO – World health organization

I. INTRODUCTION

In women, breast cancer, according to Globocan data for 2020, is the most commonly diagnosed malignancy and the most common cause of death from malignant disease, and as the leading cause of death in both sexes in the world ranks fifth (Globocan 2020). Morbidity and mortality are expected to increase in the coming years. The International Agency for Research on Cancer estimates that by 2040, the incidence of breast cancer will increase by more than a third to more than 3 million new cases a year, and mortality from the disease will increase. increased by more than half so far and deaths are expected to be more than 1 million a year.

Some genetic predispositions and a number of risk factors influence the development of breast cancer. Most hereditary factors are associated with BRCA1 and BRCA2 mutations (Park MJ et al., 2011). Risk factors are related to exposure of the parenchyma of the mammary gland to various hormones during life (early menarche, late menopause, obesity, use of hormonal drugs), reduced physical activity, smoking, alcohol use and others. (Collaborative Group, 2002; Steiner E et al., 2008; Park MJ et al., 2011). The incidence of the disease increases rapidly with age after 40 years (Berg WA, 2007; Berg WA, 2009).

The World Health Organization (WHO), based on histogenesis and growth patterns, examines different histological categories of breast cancer, which differ in their associated risk factors, clinical picture, response to treatment and prognosis, WHO Classification of Tumors Editorial Board, 2019). In addition to classical prognostic factors such as histological variant, degree of differentiation and TNM stage, treatment also depends on the molecular profile of the tumor, ie estrogen receptor (ER) expression, progesterone receptor (PR), epidermal growth receptor factor (HER2) and the proliferative marker Ki67 (Li CI et al., 2003; De Azambuja E et al., 2007; Yerushalmi R et al., 2010; Goldhirsch A et al., 2013; Makki J, 2015; Mueller C et al., 2018; Foukakis T, Bergh J, 2021).

The main therapeutic methods in patients with breast cancer are: surgery, radiation and chemotherapy, hormone and targeted therapy (Dhankhar R et al., 2010). Despite these well-established therapeutic approaches over the years, science is constantly looking for new additional strategies to better deal with this widespread disease. One of the directions we are working on is to find options for activating cell death in tumor cells. In humans, the p53 protein that induces apoptosis is dysfunctional in more than half of carcinomas. Until recently, apoptosis was considered the only form of programmed cell death, while necrosis was considered an "unexpected" form of death unregulated by molecular mechanisms (Linkermann A, Green DR, 2014). Therapeutically induced apoptosis, in most cases of mammary carcinoma, was considered the only strategy for inducing cell death. Recently, a new form of programmed necrotic cell death has been discovered, necroptosis, which is similar in mechanism to apoptosis and has morphological similarities to necrosis (Christofferson DE, Yuan J, 2010). Necroptosis is mediated mainly by Receptor Interacting Serine / Threonine Kinase 1 (RIPK1), Receptor Interacting Serine / Threonine Kinase 3 (RIPK3) and Mixed Lineage Kinase Domain Like Pseudokinase (MLKL), and is inhibited by necrostatin-1 (Nec-1), which is the first well-defined necroptosis inhibitor to primarily affect RIPK1 activity (Degterev A et al., 2008).

Necroptosis is involved in the regulation of a number of physiological processes, but also in the development of many diseases in humans. In addition to a key role in viral infections, necroptosis is thought to play important functions in regulating the biology of malignancies, such as oncogenesis, metastatic potential, tumor immunity, and more. (Stoll G et al., 2017; Seehawer M et al., 2018). As an intermediate form of cell death with characteristics of both apoptosis and necrosis, it is suggested that key mediators of the necroptotic pathway alone or in combination potentiate metastasis and progression in malignant tumors (Park S et al., 2009; Strilic B et al. ., 2016; McCormick KD et al., 2016). There is also evidence that necroptosis also serves as a mechanism that prevents the

development of a tumor process when apoptosis is compromised (Feng X et al., 2015; Höckendorf U et al., 2016).

Data in the literature on the role of necroptosis in the progression and survival of breast cancer patients are extremely scarce, and a more detailed study of its signaling pathways would clarify its involvement in mammary carcinogenesis and provide new strategies for therapeutic response.

II. PURPOUSE AND TASK

The aim of the present study was to investigate the expression of the necroptotic marker RIPK3 in relation to clinical and morphological parameters, receptor status, proliferative marker Ki67 and survival of patients without progression in breast cancer. The following tasks were formulated to achieve the set goal:

1. To study the clinical and morphological characteristics of patients with breast cancer.
2. To determine the receptor status: ER, PR, HER2 and the proliferative marker Ki67 in the tumor tissue of patients with mammary carcinoma.
3. To study the survival of patients with breast cancer.
4. To investigate the cytoplasmic and nuclear expression of RIPK3 in neoplastic tissue in breast cancer.
5. To analyze the cytoplasmic expression of RIPK3 in relation to clinical and morphological characteristics, molecular profile, proliferative marker Ki67 and survival of patients with breast cancer.
6. To study the nuclear expression of RIPK3 in relation to clinical and morphological characteristics, molecular profile, proliferative marker Ki67 and survival of patients with breast cancer.

III. MATERIALS AND METHODS

Bases for realization of the dissertation work

- Department of General and Clinical Pathology, Forensic Medicine and Deontology - MU - Varna
- Clinic of General and Clinical Pathology - University Hospital "St. Marina" - Varna
- Clinic of Medical Oncology - University Hospital "St. Marina" – Varna

Patients and characteristics of the studied groups

A total of 98 patients were included in the present study, 79 of whom were diagnosed with breast cancer and the remaining 19 were controls. The target group included 16 cases of lobular carcinoma and 63 cases of ductal carcinoma. The controls were represented by 10 cases of non-proliferative type of fibrocystic disease (PCB) and 9 cases of proliferative type. The diagnosis and stage of 71 of the cases of breast cancer have been determined according to the 5th edition of the WHO on breast tumors from 2019. These patients underwent mastectomy with regional lymph dissection. The diagnosis of the remaining 8 cases of cancer was made histologically on a tru-cut biopsy with sufficient tumor tissue, and the stage was determined by imaging. In 58 cases, subsequent hormone, radiation and / or chemotherapy was performed at the University Hospital "St. Marina" - Varna and these patients were determined disease-free survival (Disease-free survival - DFS). The level of nuclear and cytoplasmic expression of RIPK3 was reported in all patients.

Routine tests

In each case of breast cancer, an average of four to five tumor materials were taken for histological examination, and additional skin and nipple materials were available when available. All sent lymph nodes were examined. The materials were fixed in 10% neutral buffered formalin and after the appropriate treatment were embedded in paraffin with a melting point of 52ºC - 54ºC in order to prepare

paraffin blocks. Sections 5 μm thick were standardly stained with hematoxylin-eosin to assess histological changes in the tumor and for the presence of metastases in the sent lymph nodes. In all tumors the histological type, their degree of differentiation, the stage of the disease by TNM were determined, their immunohistochemical status was assessed according to the expression of ER, PR, HER2 and Ki67.

Specific research methods

Immunohistochemical method

Pre-prepared immunohistochemical specimens

After the diagnosis of each tumor, the expression levels of the immunohistochemical markers ER, PR, HER2 and Ki67 were assessed to determine its molecular profile. The antibodies used are shown in Table 1.

Table 1. *Reagents used.*

Antibody	Dilution	Positive control	Manufacturer
Monoclonal Rabbit Anti-Human Estrogen Receptor α, Clone EP1	Ready for use	FCD, proliferative type	Dako
Monoclonal Mouse Anti-Human Progesterone Receptor, Clone PgR 636	Ready for use	FCD, proliferative type	Dako
Polyclonal Rabbit Anti-Human c-erbB-2 Oncoprotein, Unconjugated, Affinity isolated	1:600	Positive breast cancer	Dako
Monoclonal Mouse Anti-Human Ki-67 Antigen, MIB-1	Ready for use	Tonsilla	Dako

Immunohistochemical studies performed for the purposes of the dissertation

An indirect immunoperoxidase method was used for immunohistochemical analysis using the mini KIT high Ph DAKO

K8024. Antibodies, staining reagents and operating concentrations used are presented in Tables 2 and 3.

The antibody Anti-RIP3, cat. № ab62344. The antibodies are manufactured by ABCAM's RabMab technology.

Negative controls were designed by incubating sections of the paraffin blocks used with normal non-immune serum instead of the primary antibody.

Liver tissues stained with Anti-RIPK3 were used for positive controls.

Table 2. *Reagents used.*

Antibody	Dilution	Positive control	Marker for:	Manufacturer
Anti RIPK3(ab62344) Rabbit polyclonal to RIP3	1:100	Liver	Necroptosis	ABCAM's RabMab technology

Table 3. *Staining systems and other reagents.*

HRP- DAB System	Original staining system	Dako
Mayer's hematoxilin	Counterstaining	Dako

RIPK3 expression levels and interpretation of results

Immunohistochemical expression of RIPK3 was assessed semiquantitatively using H-score (histo-score) on tissue sections. First, the intensity of cytoplasmic expression was assessed for each cell in different fields (0 no response, 1+ weak positivity, 2+ moderate positivity or 3+ strong positivity). The percentage of positive cells for each intensity was determined, and finally the H-score was calculated using the following formula: [1x (% cells with 1+) + 2x (% cells with 2+) + 3x (% cells with 3+)], in the range 0 to 300.

The H-score was also used to assess the nuclear expression of RIPK3. The result was obtained by the formula:

[1x (% cores with 1+) + 2x (% cores with 2+) + 3x (% cores with 3+)], in the range from 0 to 300.

Statistical methods

The data were processed and analyzed using SPSS ver software. 23. Mann-Whitney test, Chi-square ($\chi 2$) test, correspondence analysis, Kruskal-Wallis test and comparison of Pairwise Comparisons were used to compare and evaluate correlations between RIPK3 expression in neoplastic tissue and clinical and pathological characteristics of patients, such as age, histological type of tumor, T-stage, degree of differentiation, degree of tumor spread (metastasis), expression of immunohistochemical markers ER, PR, HER2 and Ki67 (separately for every one of them).

In order to process the data with the listed tests, the values of nuclear and cytoplasmic expression of RIPK3 in tumor tissue were distributed relative to the median and percentiles (up to the 33rd percentile - low expression, between the 33rd and 66th percentile - moderate expression and above 66th percentile - high expression).

The Shapiro-Wilk test was used to study the normality of the distribution (test of normality).

Qualitative variables are presented with an absolute number and relative share in the descriptive analysis of the demographic and clinical characteristics of the participants in the study, and quantitative - with average and standard deviation.

The $\chi 2$ test was used to test hypotheses for the relationship between two qualitative variables. Spearman's rank correlation was used to study the relationship between two quantities measured on the ordinal scale.

Comparisons between more than two groups were performed with the Kruskal-Wallis one way ANOVA test.

The strength of the dependence between two variables was estimated by the coefficient Kendall's tau c. Its values are estimated as follows:

- $<\pm$ 0.1 - very weak dependence

- from ± 0.1 to ± 0.19 - weak dependence
- from ± 0.2 to ± 0.29 - moderate dependence
-> ± 0.3 - strong dependence

Survival curves according to the expression of RIPK3 in tumor tissue were assessed using the Kaplan-Meier method, and differences were measured using the Logrank test.

Each indicator is represented by a 95% Confidence Interval (CI).

The results were reported as statistically significant at $p<0.05$.

The results are presented in summary form in tables and are illustrated with appropriate graphs: columnar, point, linear and others.

IV. RESULTS AND DISCUSSION

Clinical and morphological characteristics of patients with breast cancer

Age of patients with breast cancer

79 patients with a diagnosis of breast cancer operated on at the University Hospital "St. Marina EAD for a period of 8 years (2010-2018). The mean age of patients was 59.38 ± 12.2 years, with a minimum age of 27 years and a maximum age of 82 years (Figure 1).

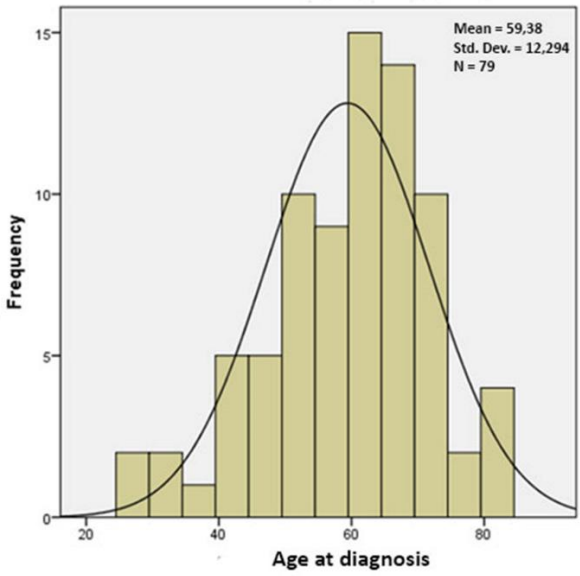


Figure 1. *Frequency of age distribution of patients with breast cancer.*

Our results (Figure 2) show that the largest number of patients with breast cancer - 32 (40.51%) cases are in the age group - 61-70 years.

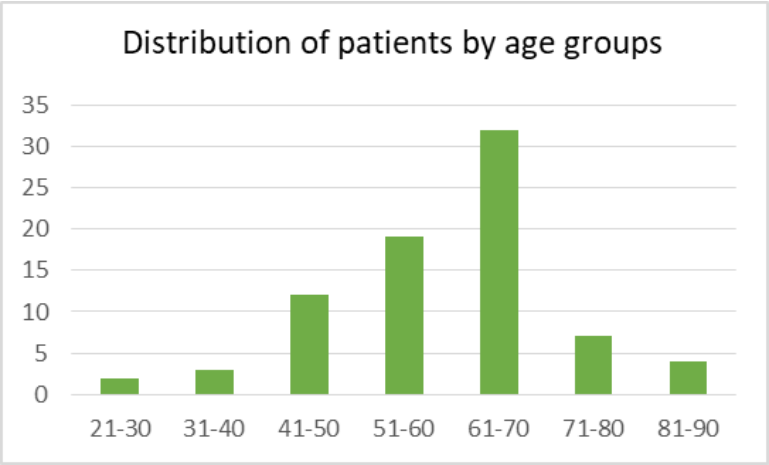


Figure 2. *Distribution of patients by age groups.*

For the subsequent statistical processing of patients' age data, the cases were divided into two groups: ≤65 years (52 patients, representing 65.82% of all 79 cases) and> 65 years (27 patients - 34.18%) (Figure 3).

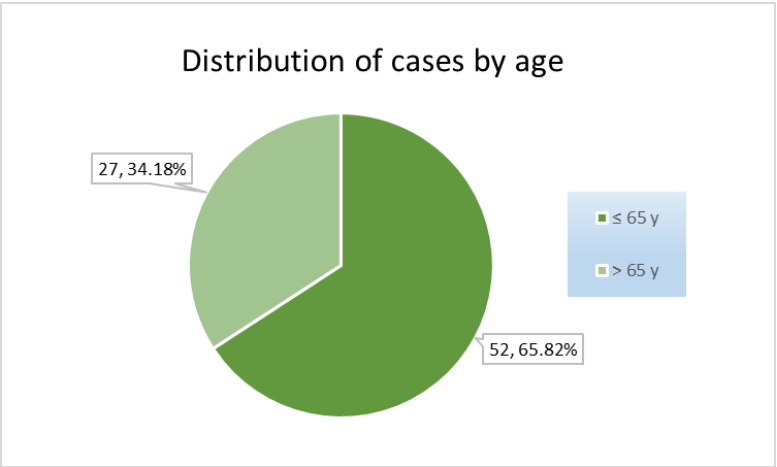


Figure 3*. Distribution of cases by age.*

Histological type of tumor

Of a total of 79 breast cancer cases studied, 63 (79.75%) were diagnosed with ductal carcinoma of the non-special type (NOS), and the remaining 16 (20.25%) were categorized as lobular carcinoma (Figure 4-6).

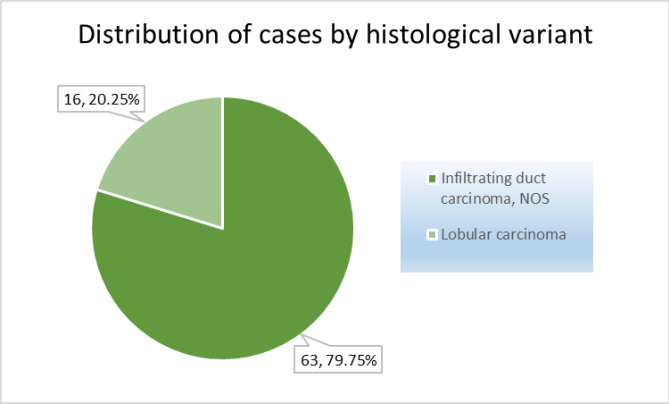


Figure 4. *Distribution of cases by histological variant*

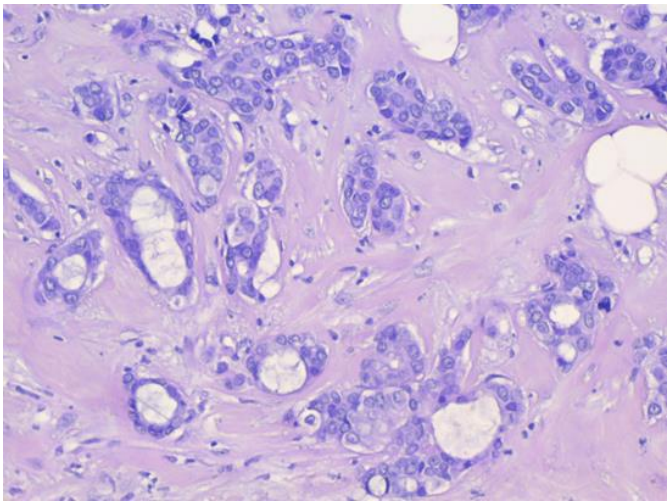


Figure 5. *Infiltrating duct carcinoma (NOS), HE x200.*

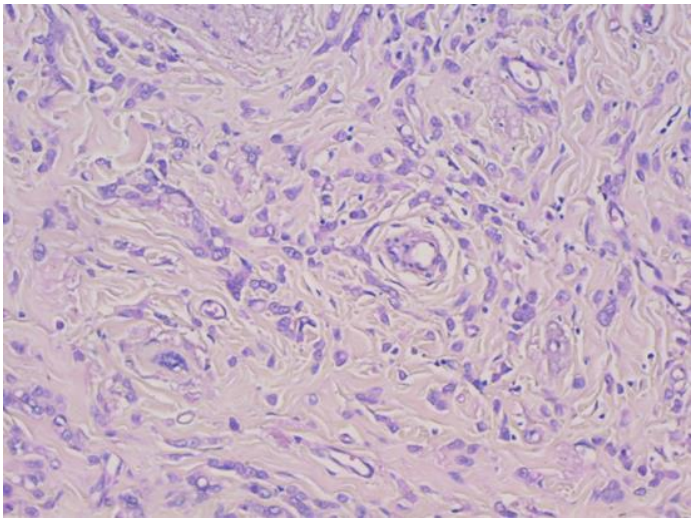


Figure 6. *Lobular carcinoma of the mammary gland, HE x200.*

T-stage of breast cancer

Of the 79 cases of breast cancer studied, only one (1.27%) patient had a T3 stage of the tumor. In stage T2 were 42 (53.16%) of the cases, in stage T1 were 33 (41.77%) of the cases and stage T4 was found in 3 (3.80%) patients (Figure 7).

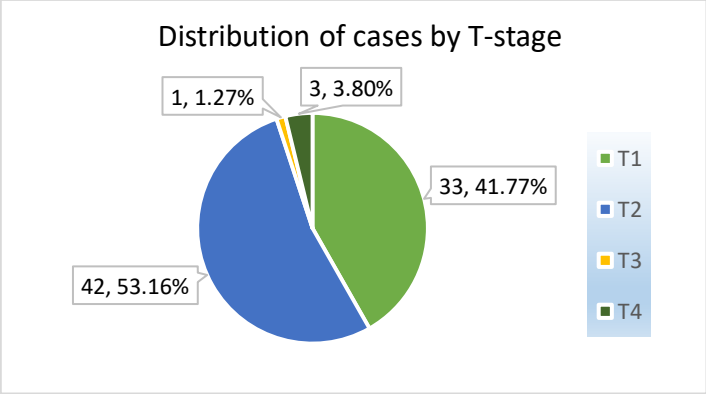


Figure 7. *Distribution of cases by T-stage*

Degree of differentiation of breast cancers

According to the degree of differentiation of tumors, they are distributed as follows: highly differentiated tumors (G1) - 17 (21.52%) cases, moderately differentiated tumors (G2) - 33 (41.77%) cases and poorly differentiated tumors (G3)) - 29 (36.71%) cases (Figure 8). Our results regarding the distribution of breast cancer cases in relation to the degree of differentiation do not differ from the data of Won et al. (2021), which determined the degree of differentiation of breast carcinomas in 257 patients. They, like us, found that the largest relative share of moderately differentiated tumors, G2 (51.2%), followed by low-differentiated tumors, G3 (29.9%) and the lowest number of cases with highly differentiated tumors, G1 (18.9%). Taken together, data from the literature and those from the present study show that breast cancer most often shows a moderate degree of differentiation at the time of diagnosis.

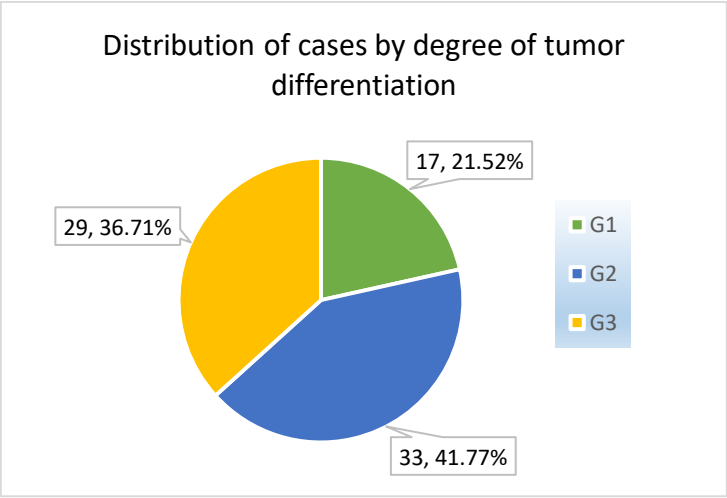


Figure 8. *Distribution of cases by degree of tumor differentiation.*

Metastases

According to their prevalence, the tumors we studied were grouped into three categories (Figure 9). The first group includes

tumors located only in the breast, without the presence of metastases. 31 (40.26%) cases are included in this group. The second group consists of 30 (38.96%) cases of locally advanced carcinomas with metastases in regional lymph nodes. The third group includes 16 (20.78%) cases in which distant metastases are detected. For two of the patients there is no information about the N and M stages of the disease. The model of the spread of the tumor process established by us during its diagnosis does not differ from the data in the study of Won et al. (2021), in which patients with non-metastatic disease (N0) also predominate, accounting for 146 (56.8%) of all 257 cases. In second place in terms of frequency is the group with locally advanced disease (N1, N2 and N3), represented by 107 cases (41.6%) and only 4 (1.6%) are patients with distant metastases.

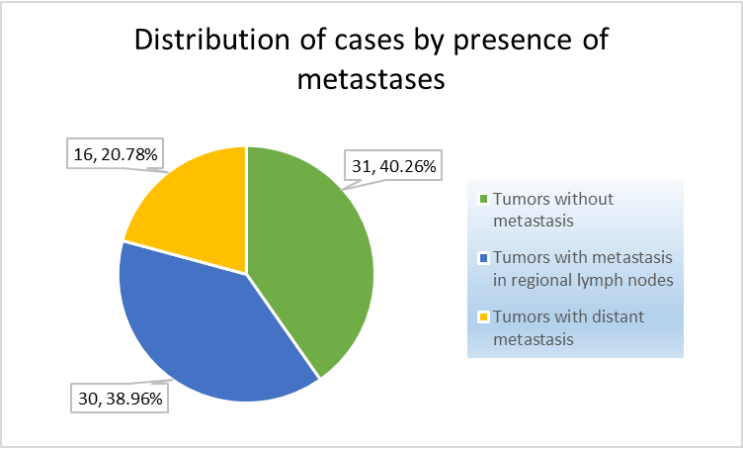


Figure 9. *Distribution of cases according to N and M stages.*

Receptor status and Ki67 in tumor tissue in breast cancer

Receptor status of breast cancer

According to the expression of ER, PR and HER2 tumors are divided into three groups as follows: ER-positive tumors - 32 (40.51%) cases, of which 30 are PR-positive and the other two are PR-negative, HER2 -positive tumors - 26 (32.91%) cases and triple-

negative carcinomas (ER-, PR-, HER2-negative) - 21 (26.58%) cases (Figure 10 - 13).

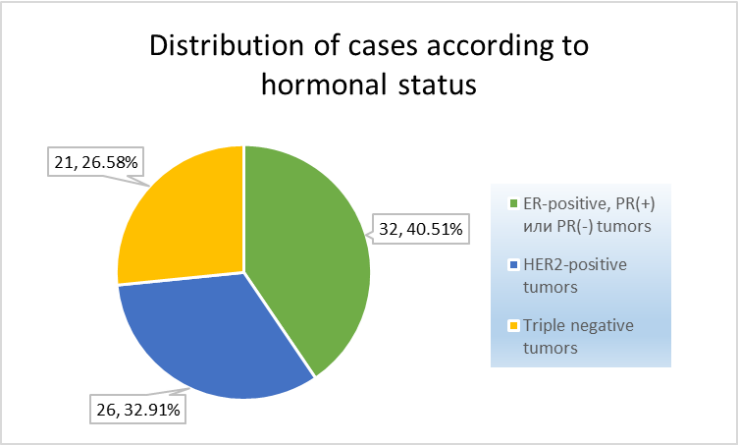


Figure 10. *Distribution of cases according to hormonal status.*

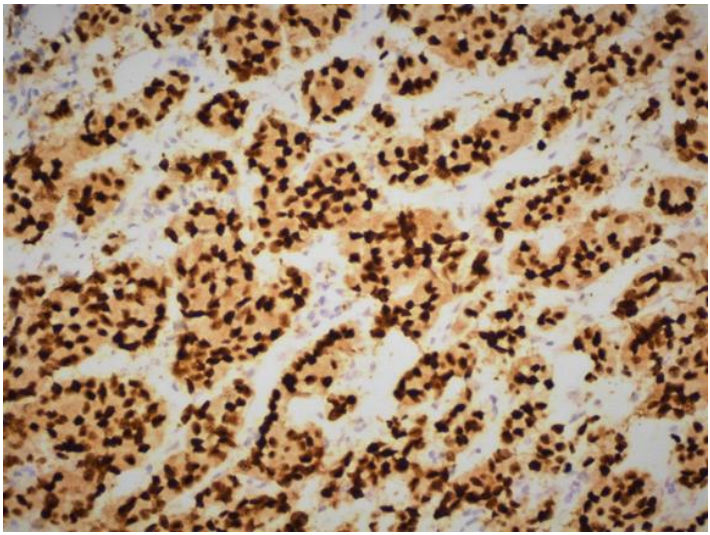


Figure 11. *Immunohistochemical expression of ER in ER-positive tumor, x 200.*

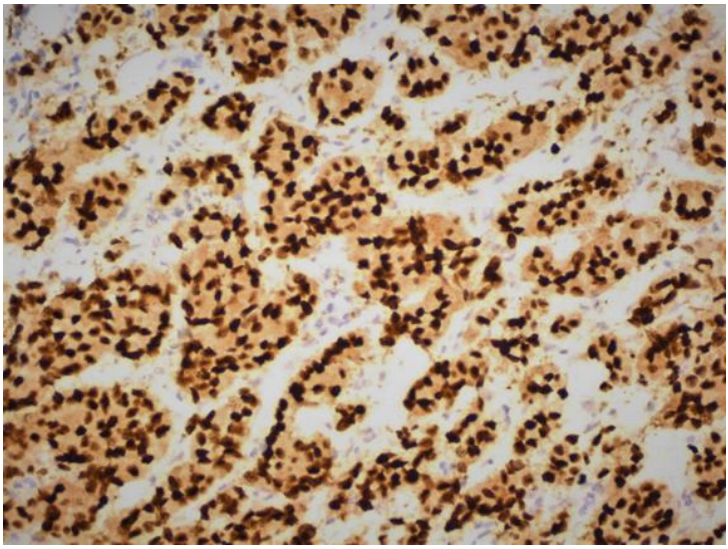


Figure 12. *Positive immunohistochemical expression of PR, x 200.*

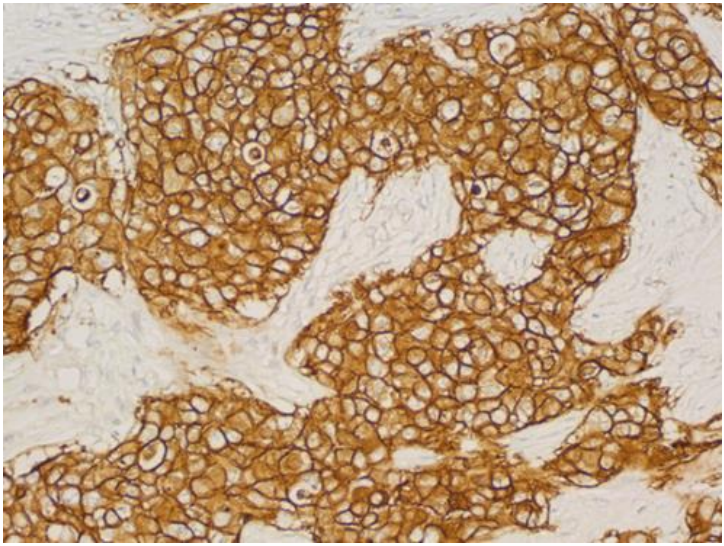


Figure 13. *Positive immunohistochemical expression of HER2, x 200.*

Expression of Ki67 in tumor tissue in breast cancer

According to this indicator, the cases are categorized into two groups: carcinomas with Ki67 <14%, which are 26 cases (32.91%) and a group with Ki67≥14%, 53 cases (67.09%) (Figure 14 - 16).

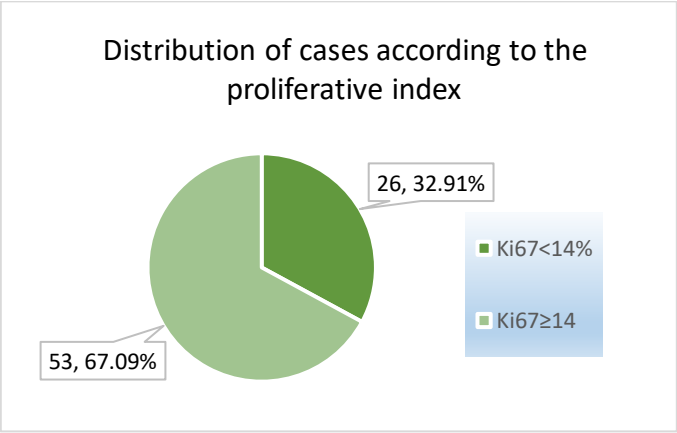


Figure 14. *Distribution of cases according to the index of proliferative activity.*

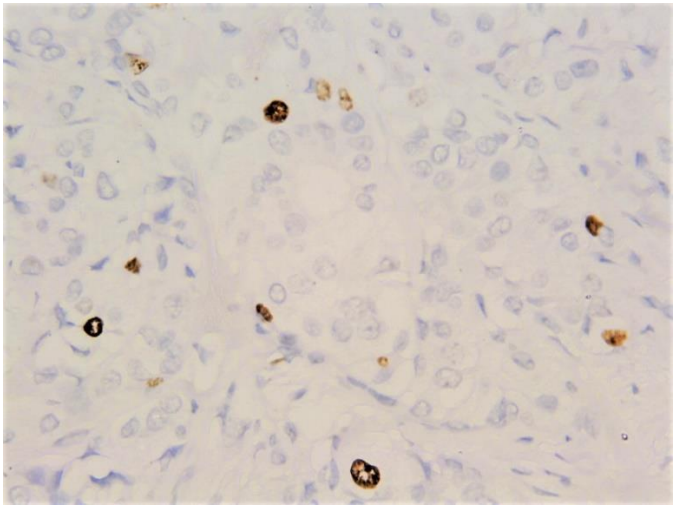


Figure 15. *Immunohistochemical expression of Ki67 <14%, x 400.*

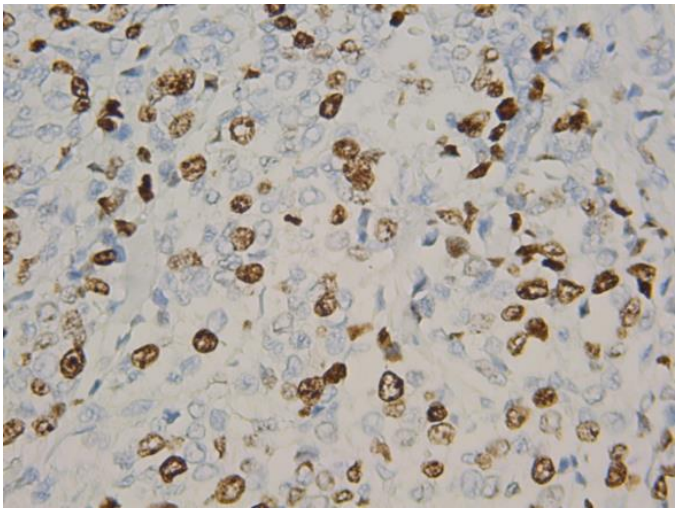


Figure 16. *Immunohistochemical expression of Ki67≥14%, x 400.*

Progression-free survival in patients with breast cancer

Progression-free survival of breast cancer patients by month is shown in Figure 17. Of the 79 cases of breast cancer studied, survival data were available in 58 patients. All of them underwent adjuvant hormone, radiation and / or chemotherapy. After treatment, remission was achieved and data on the course of the disease were available after diagnosis and therapy.

The median progression-free survival in the current patient sample was 113.8 months (CI, 102.5 - 125.1).

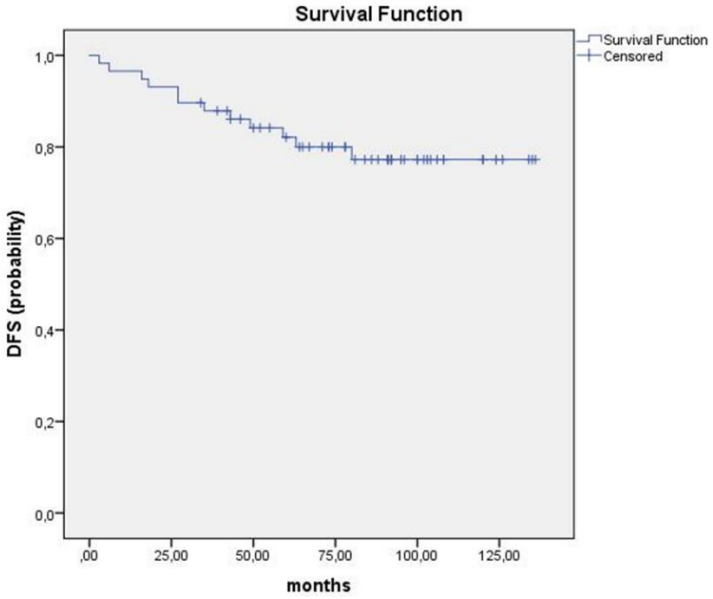


Figure 17. *Kaplan-Meier curve of disease-free survival distribution in patients with breast cancer in months.*

The cytoplasmic and nuclear expression of RIP3 was examined in the tumor tissue of all 79 patients. The mean cytoplasmic expression of RIP3 determined by H-score was 119.6 ± 56.4, with a minimum of 5 and a maximum of 230 (Figure 18). The mean RIP3 nuclear expression was 189.4 ± 54.2, with a minimum of 5 and a maximum of 285 (Figure 19).

The mean values of cytoplasmic expression in the present study did not differ significantly from the mean values of cytoplasmic expression of RIP3 in colorectal cancer, where they were 131.88 ± 84.00, also determined by H-score (Stefanova N., 2017). With regard to the average values of nuclear expression in the same study, values of 132.37 ± 91.05 were reported and they are significantly lower than the data obtained in the present study.

The Shapiro-Wilk test was used to verify the normal distribution of nuclear and cytoplasmic RIP3 expression. The obtained values of p = 0.014 for cytoplasmic expression and p = 0.004 for nuclear expression show a significant difference in the distribution of cases. This means that the intensity of the RIP3 response in the nucleus and cytoplasm of tumor cells in individual cases does not follow the normal distribution (Figures 18 and 19).

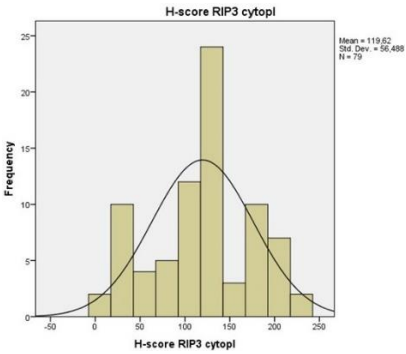


Figure 18. Cytoplasmic expression of RIP3 in tumor tissue of breast cancer.

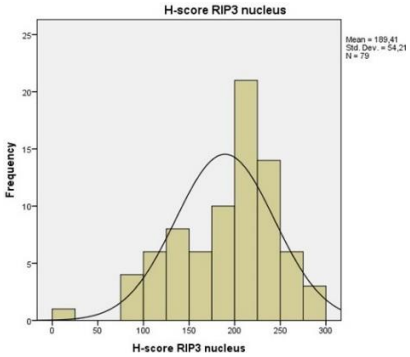


Figure 19. Nuclear expression of RIP3 in tumor tissue of breast cancer.

The values of nuclear and cytoplasmic expression of RIP3 in tumor tissue relative to the median and percentiles were also determined. The median immunohistochemical response in the cytoplasm of tumor cells is 120 and that in the nucleus is 200. The values of nuclear and cytoplasmic expression of RIP3 up to the 33rd percentile, between the 33rd and 66th percentiles and above the 66th percentile are respectively : up to 102.00, between 102.00 and 139.00 and between 139.00 and 230.00 for cytoplasmic expression and up to 182.60, between 182.60 and 215.00 and between 215.00 and 285.00 for nuclear expression.

In 19 patients with PKB, divided into two groups (10 cases with non-proliferative type PKB and 9 cases with proliferative type PKB), the expression of RIP3 was studied, and cytoplasmic and nuclear reactions were found in both types of PKB (Figures 20, 21).

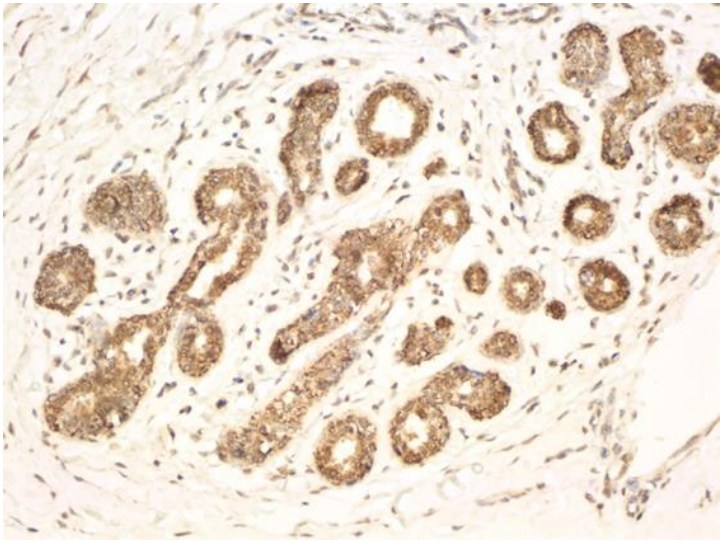
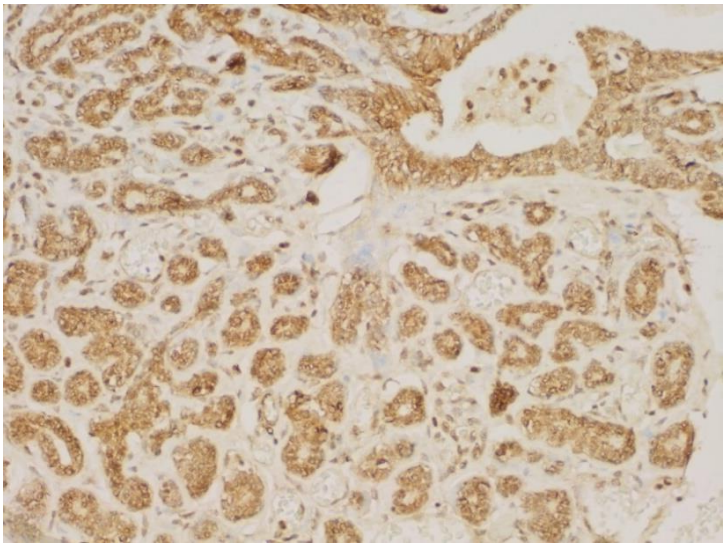


***Figure 20**. Immunohistochemical expression of RIPK3 in non-proliferative type FCBD, x200.*



***Фигура 21.** Immunohistochemical expression of RIPK3 in proliferative type FCBD, x200.*

The mean value of cytoplasmic expression of RIP3 in the group of proliferative type FCB is 180.6 ± 8.4, the minimum is 170 and the maximum is 195. In cases of non-proliferative type FCB the levels of expression in the cytoplasm are as follows: mean - 187.5 ± 10.1, minimum - 170 and maximum - 200. No significant difference was found between the cytoplasmic expression of the necroptosis marker in the two groups of patients (p> 0.05) (Figure 22).

The analysis of the cytoplasmic expression of RIPK3 in the non-tumor tissue adjacent to colorectal cancer, based on the mean values, showed lower levels of expression - 149.38 ± 92.00 (N. Stefanova (2017)).

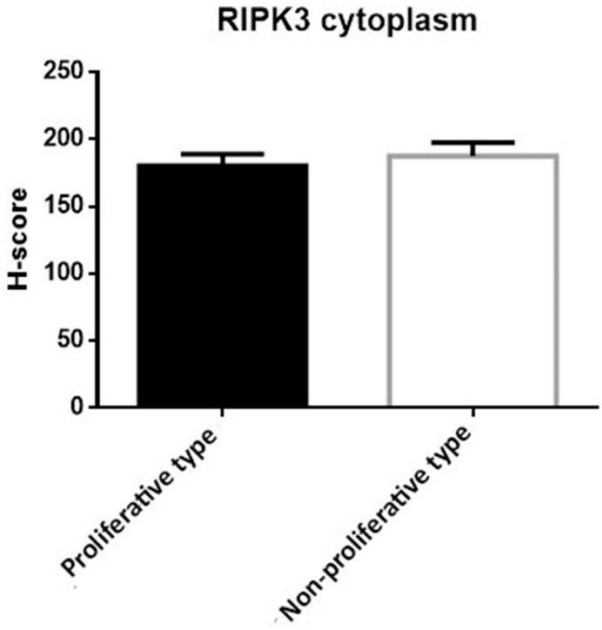


Figure 22. *Cytoplasmic expression of RIPK3 in proliferative and non-proliferative type FCB.*

The mean values of nuclear expression of RIP3 in the tissues of the proliferative type FCB is 83.6 ± 11.7, with a minimum of 60 and a maximum of 95. The results are similar in value in the group of nonproliferative type FCB, where the average is 87 , 5 ± 17.6, the minimum is 60 and the maximum is 115 (Figure 23).

Regardless of the histological variant of FCB, nuclear expression in breast tissue is lower compared to nuclear expression in non-tumor tissue of colorectal cancer, where the mean values are 135.94 ± 94.15 (N. Stefanova, 2017).

In our opinion, the differences in cytoplasmic and nuclear expression in PCBs and non-tumor tissue in colorectal cancer may be tissue-related.

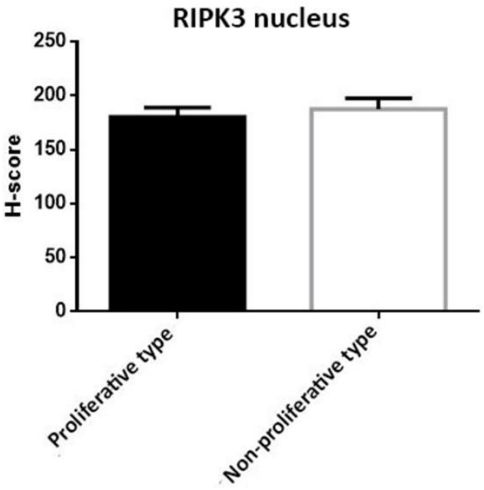


Figure 23. *Nuclear expression of RIPK3 in proliferative and non-proliferative type FCB.*

In view of the data presented on the cytoplasmic and nuclear expression of RIPK3 for PKB, it was considered that the two groups of PKB patients could be pooled and considered together. Subsequent analyzes between the expression of RIPK3 in tumor tissue and in the

control group took the mean cytoplasmic and nuclear expression of RIPK3 for all patients with PKB, which were 184.2 ± 9.7 and 85.6 ± 14, respectively.

Comparative analysis between the mean values of RIP3 expression in tumor tissue and in the control group

The analysis of the mean values of cytoplasmic and nuclear expression of RIP3 in tumor tissue and in the control group showed a statistically significant difference in both types of expression (p <0.0001).
Figure 24 shows the H-score of the cytoplasmic expression of RIPK3 in mammary carcinoma tumor tissue and in the control group. In PCB tissues, expression is higher than in tumor tissue.

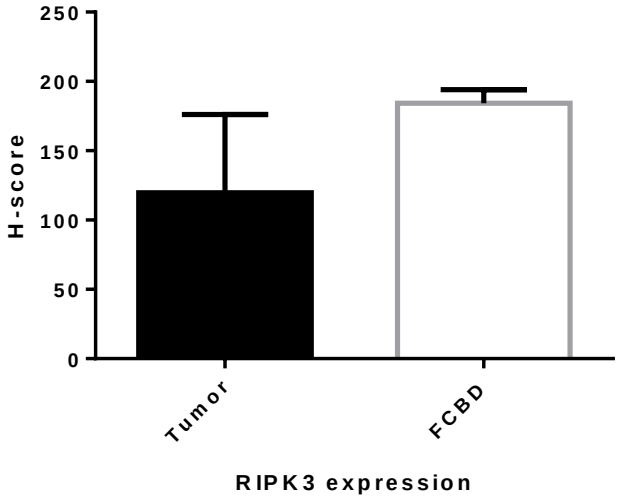


Figure 24. *Cytoplasmic expression of RIPK3 in tumor tissue and in FCB.*

The data on nuclear expression are completely opposite (Figure 25). Nuclear expression of RIPK3 in tumor tissue was significantly higher than in the control group.

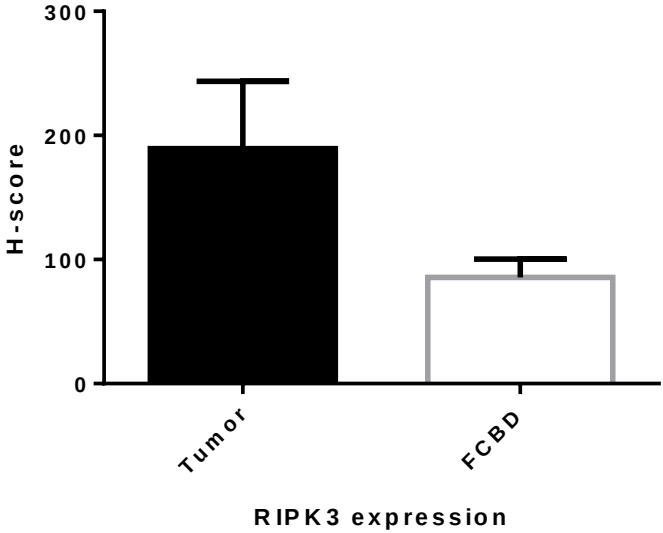


Figure 25. *Nuclear expression of RIPK3 in tumor tissue and in FCB.*

Our data differ from the results published by Stefanova (2017) for colorectal cancer, in which no statistically significant difference was found in the two localizations of immunohistochemical expression of RIPK3, nuclear and cytoplasmic, between tumor and non-tumor tissue, when a three-point rating scale is used: "-", "+", "++". Our results do not differ from the data of Feng et al. (2015) who evaluated the immunohistochemical cytoplasmic expression of RIPK3 in tumor tissue of 112 patients with colorectal cancer and compared it with normal mucosa, also using three stages of evaluation. They found a higher cytoplasmic level of RIPK3 expression in non-tumor tissue than in tumor tissue. A similar decrease in TIPK3 tissue expression in colorectal cancer compared to neighboring non-tumor tissue has been reported by other authors (Moriwaki et al., 2015). In addition to colon carcinoma, lower levels of RIPK3 expression were observed in CD34 + blasts from patients with acute myeloid leukemia compared to CD34 + bone marrow cells from healthy donors using RQ-PCR (Nugues AL

et al., 2014). Results similar to the present study were found when analyzing the tissue expression of RIPK3 by Western blot (Karami-Tehrani et al., 2016). The authors determined the level of RIPK3 in 30 cases of breast cancer and compared it with an adjacent normal breast parenchyma in 20 of the patients and found significantly lower levels of RIPK3 expression in the tumor. Our results and literature data suggest that tumors, in the course of neoplastic progression, have acquired mechanisms by which they can avoid necroptosis by reduced expression of key components of the signaling pathway of this type of cell death.

Comparative analysis between nuclear and cytoplasmic expression of RIPK3 in tumor tissue

To investigate whether there is a relationship between nuclear and cytoplasmic RIPK3 expression in tumor tissue, we applied correspondence analysis. Table 4 shows the distribution of cases according to the RIPK3 response in the nucleus and cytoplasm of tumor cells by percentiles. These data are presented graphically in Figure 26, in which the distance between the points of the individual categories reflects the relationship between them. The closer two points in the coordinate system are located, the stronger the relationship between them. Figure 26 shows that the dots are far apart, which means that there is no relationship between nuclear and cytoplasmic RIPK3 expression in tumor tissue. The comparative analysis shows that there is no significant relationship between the studied indicators ($p = 0.926$).

Table 4. *Distribution of cases according to nuclear and cytoplasmic expression of RIPK3 in tumor tissue by percentiles.*

Group	**RIPK3 (low nucl. expr.)**	**RIPK3 (moderate nucl. expr.)**	**RIPK3 (high nucl. expr.)**	**P-value**
RIPK3 (low cytopl. expr.)	10 (38,5%)	8 (28,6%)	8 (32,0%)	**0,926**
RIPK3 (moderate cytopl. expr.)	7 (26,9%)	10 (35,7%)	9 (36,0%)	
RIPK3 (high cytopl. expr.)	9 (34,6%)	10 (35,7%)	8 (32,0%)	
Total	**26 (100%)**	**28 (100%)**	**25 (100%)**	

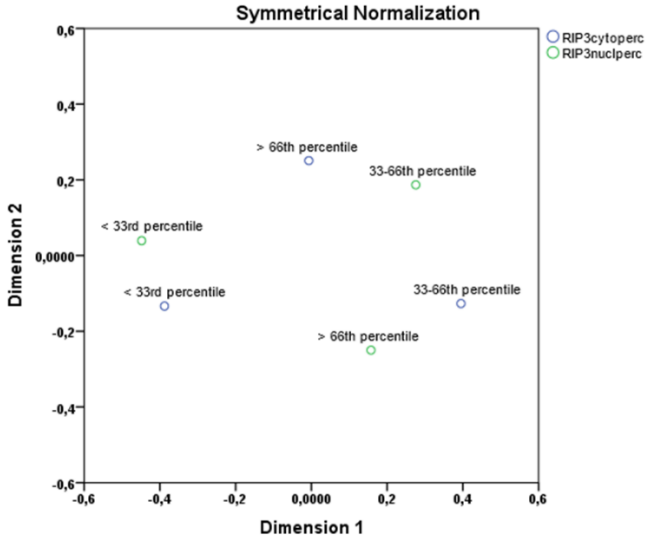


Figure 26. *Dependence between nuclear and cytoplasmic expression of RIPK3 in tumor tissue.*

Comparative analysis of cytoplasmic expression of RIPK3 depending on clinical and morphological characteristics, molecular profile, proliferative marker Ki67 and survival of patients with breast cancer

Comparative analysis of cytoplasmic expression of RIPK3 depending on the clinical and morphological characteristics of the tumor

Comparative analysis of the cytoplasmic expression of RIPK3 according to the age of the patients

The Chi-square (χ^2) test was used to analyze the data to assess the relationship between cytoplasmic RIPK3 expression and the age of the patients. The value of the obtained p = 0.639 shows that there is no relationship between the two indicators: the level of cytoplasmic expression of RIPK3 and the distribution of patients by age (above and below 65 years) (Table 5).

Table 5. Age *distribution of patients by median cytoplasmic expression of RIPK3.*

Group	**RIPK3 (low expression)**	**RIPK3 (high expression)**	**P-value**
≤ 65 y	27 (67,5%)	25 (64,1%)	**0,639**
> 65 y	13 (32,5%)	14 (35,9%)	
Total	**40 (100%)**	**39 (100%)**	

The Mann-Whitney assay, used to analyze the cytoplasmic expression of RIPK3 in relation to the age of the patients, again confirmed the absence of a significant relationship between the two parameters (p = 0.992). The mean value of cytoplasmic expression of RIPK3 in the group of patients under 65 years of age was 119.4 ± 55.14, and in the remaining 27 patients over 65 years it was almost the same - 120.0 ± 60.08 (Table 6) .

Table 6. *Relationship between cytoplasmic expression of RIPK3 and age of patients.*

Group	Cases (n)	Mean cytoplasmic expression of RIPK3	Standard deviation	P-value
≤ 65 y	52	119,4	55,14	**0,992**
> 65 y	27	120,0	60,08	

Won et al. (2021) looked for a relationship between the immunohistochemical expression of RIPK3 in the cytoplasm of tumor cells and the age of 257 patients with breast cancer and, like us, found no relationship between the two indicators. In their study, patients are divided into two groups, but they accept 50 as the limit. The data from the current study do not differ from those of Stefanova (2017), which analyzes the two indicators of colorectal cancer and similarly divides patients into two groups: under 65 and over 65 in colorectal cancer, Feng et al. (2015) also found no significant relationship between cytoplasmic expression of RIPK3 and age, with the limit again being 65 years. In the literature there are data different from those presented so far. Karami-Tehrani et al. (2016), divide patients with breast cancer into three age groups: under 40 years, between 40 and 60 years and over 60 years. They found statistically significant higher expression of RIPK3 in tumors when patients are over 60 years. , compared to the other two groups - under 40 and between 40 and 60. In our opinion, the differences in the age indicator are probably due to the random selection of patients in the studies and depend on the chosen method of distribution by groups.

Comparative analysis of cytoplasmic expression of RIPK3 depending on the histological type of the tumor

The comparative analysis of the cytoplasmic expression data of RIPK3 with respect to the histological variant of the tumor was performed using the χ^2 test. The value of the obtained $p = 0.082$ shows

that there is a tendency for a relationship between the levels of cytoplasmic expression of RIPK3 and the histological variant of the tumor, but the difference is not statistically significant (Table 7). In lobular carcinoma, cases of high expression predominate, while in ductal carcinoma, low expression is more common.

Table 7. *Distribution of tumors by histological variant relative to cytoplasmic RIPK3 expression by median.*

Group	**RIPK3 (low expression)**	**RIPK3 (high expression)**	**P-value**
Infiltrating duct carcinoma (NOS)	35 (87,5%)	28 (71,8%)	**0,082**
Lobular carcinoma	5 (12,5%)	11 (28,2%)	
Total	**40 (100%)**	**39 (100%)**	

A comparative analysis of the values of cytoplasmic RIPK3 expression versus histological variant of the tumor using the Mann-Whitney test revealed a statistically significant difference in RIPK3 expression between ductal and lobular carcinoma (p = 0.046). The mean cytoplasmic expression of RIPK3 in the 16 cases of lobular carcinoma was 147.2 ± 52.19, which was significantly higher than the other 63 patients with ductal carcinoma, in whom the values were 112.6 ± 55 , 76 (Table 8).

Table 8. *Relationship between cytoplasmic RIPK3 expression and histological variant of the tumor.*

Група	**Cases (n)**	**Mean cytoplasmic expression of RIPK3**	**Standard deviation**	**P-value**
Infiltrating duct carcinoma (NOS)	63	112,6	55,76	**0,046**
Lobular carcinoma	16	147,2	52,19	

Comparative analysis of cytoplasmic expression of RIPK3 depending on the T-stage of tumors

The comparative analysis between the cytoplasmic expression of RIPK3 and the T-stage of the tumors included in the study was initially performed by χ^2 test, which did not show a relationship between the two studied indicators (p = 0.695) (Table 9).

Table 9. *Distribution of T-stage cases versus median cytoplasmic expression of RIPK3.*

Group	RIPK3 (low expression)	RIPK3 (high expression)	P-value
T1	17 (42,5%)	16 (41,0%)	**0,695**
T2	20 (50,0%)	22 (56,4%)	
T3	1 (2,5%)	0 (0,0%)	
T4	2 (5,0%)	1 (2,6%)	
Total	**40 (100%)**	**39 (100%)**	

Analysis of data from the Mann-Whitney test to assess the relationship between cytoplasmic expression of RIPK3 and T-stage tumors was performed only for cases diagnosed in T1 and T2 stages. The found p = 0.608 shows no statistically significant relationship between the two studied indicators. The mean values of cytoplasmic expression of the necroptosis marker in the 33 cases with T1 stage were 116.2 ± 61.42, and in 42 of the cases with T2 stage were 123.5 ± 53.13 (Table 10). Mean cytoplasmic RIPK3 expression values of 106.7 ± 73.71 were determined for the three T4 stage tumors, whereas for the only case of T3 stage breast cancer, averaging was not possible. Due to the small number of cases in the last two categories, they were not included in the Mann-Whitney test (Fig. 27).

Table 10. *Relationship between cytoplasmic RIPK3 expression and T-stage of tumors.*

Group	Cases (n)	Mean cytoplasmic expression of RIPK3	Standard deviation	P-value
T1	33	116,2	61,42	**0,608**
T2	42	123,5	53,13	

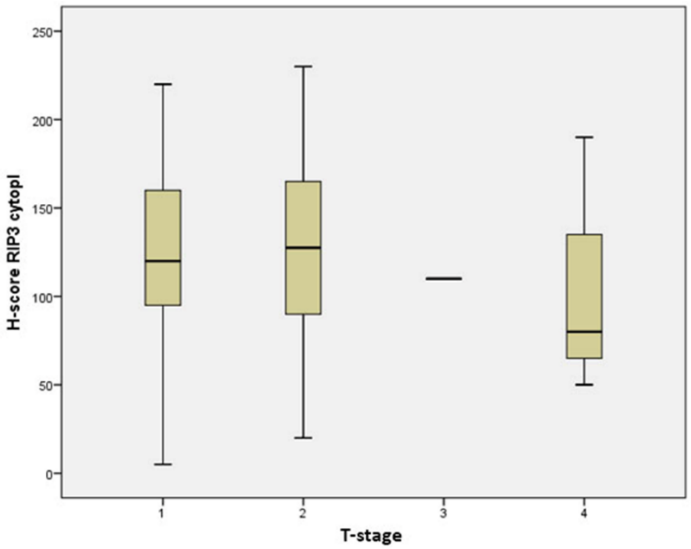


Figure 27. *Distribution of T-stage cases relative to mean cytoplasmic RIPK3 expression in tumor cells.*

When comparing the own results with those of the study by Won et al. (2021) for the relationship between the level of cytoplasmic expression of RIPK3 and the T-stage of tumors, we found that there are some similarities in the data obtained from the two studies. Won et al. (2021) reported that tumor size showed an inverse relationship with cytoplasmic expression of the marker of necroptosis in tumor cells. According to their data, large tumor sizes are associated with low RIPK3 expression (Won KY et al., 2021). The authors of the study analyzed 13 cases of breast cancer in T3 and T4 stages from a total of 257 patients and found that 2 (1.8%) of them had high cytoplasmic expression in the tumor, while the remaining 11 (7.5%)) the patient's expression is low. In the present study, of the 79 patients included, 4 were in T3 and T4 stages, with 3 (7.5%) having low cytoplasmic RIPK3 expression and 1 (2.6%) having high cytoplasmic expression. table 15) - data that do not differ from those of Won et al. (2021). Due to the extremely small number of patients in T3 and T4 stages, there is no possibility for statistical analysis of the data in the present study.

The results of Feng et al. (2015) in colorectal cancer do not differ from those of Won et al. (2021) in mammary carcinoma. Feng et al. (2015) also found that colorectal carcinomas in stage T3 and T4 have low cytoplasmic expression of RIPK3 in tumor cells. According to Karami-Tehrani et al. (2016), however, there is no significant difference between the level of RIPK3 expression in tumor cells and the size of mammary carcinoma. In a study by Karami-Tehrani et al. (2016), the size of tumors is divided into two categories: less than 2.5 cm and more than 2.5 cm, and not according to the T-stage of the disease. Similar to Karami-Tehrani et al. (2016) results have also been observed in colorectal cancer (Stefanova 2017). Stefanova also did not find a statistically significant difference in the expression of RIPK3 in colon carcinomas that are in different T-stages. In our opinion, the variations in the results may be due on the one hand to the different number of patients with carcinomas included in the individual T-stages, and on the other hand to be related to the type of tumors studied.

Comparative analysis of cytoplasmic expression of RIPK3 depending on the degree of tumor differentiation (G)

The Kruskal-Wallis test to assess the relationship between cytoplasmic RIPK3 expression in tumor cells and the degree of carcinoma differentiation showed a statistically significant difference ($p = 0.034$). From the analysis it is clear that the cytoplasmic expression of RIPK3 differs between groups of tumors with different degrees of differentiation (G1, G2 and G3) (Figure 28, Table 11).

Table 11. *Relationship between cytoplasmic expression of RIPK3 and the degree of tumor differentiation.*

Group	Cases (n)	Mean cytoplasmic expression of RIPK3	Standard deviation	P-value
G1	17	146,2	50,70	**0,034**
G2	33	124,7	50,06	
G3	29	98,3	60,21	

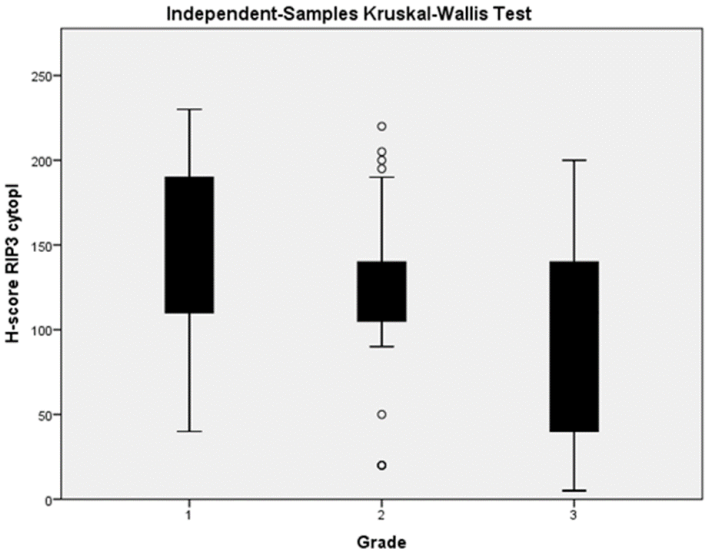


Figure 28. *Distribution of cases according to their degree of differentiation compared to the mean values of cytoplasmic expression of RIPK3 in tumor cells.*

In the subsequent additional analysis of the data to assess the relationship between tumors with varying degrees of differentiation in relation to RIPK3 expression, the groups were compared in pairs (Pairwise Comparisons). Table 11 shows the mean cytoplasmic expression of RIPK3 in G1, G2 and G3 carcinomas. Tumors with a high degree of differentiation had mean RIPK3 expression values of 146.2 ± 50.70 (Figure 29) and were significantly higher than tumors with a low degree of differentiation (mean values of 98.3 ± 60.21; = 0.011) (Figure 30). Tumors with G3 showed only a tendency to lower expression of the cytoplasmic necroptotic marker compared to tumors with G2 (124.7 ± 50.06; p = 0.104). No significant difference in cytoplasmic RIPK3 expression was found between tumors with high and moderate differentiation (p = 0.225).

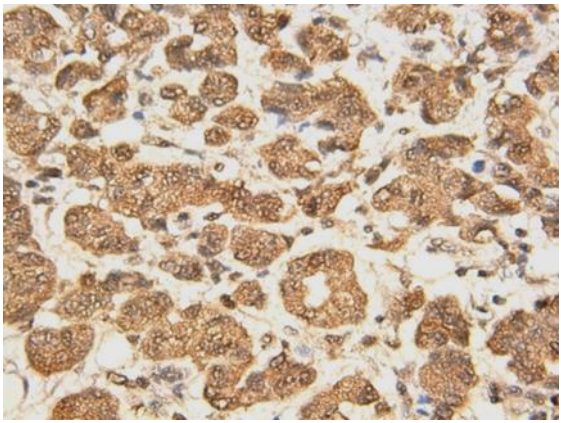


Figure 29. *High cytoplasmic expression of RIPK3 in highly differentiated tumors (G1), x 400.*

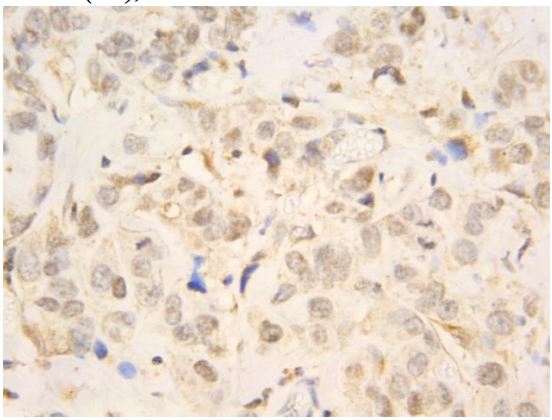


Figure 30. *Low cytoplasmic expression of RIPK3 in poorly differentiated tumors (G3), x 400.*

Won et al. (2021) found that low cytoplasmic expression of RIPK3 in mammary carcinoma was associated with severe nuclear atypism. The results obtained by us that the low expression of RIPK3 in the cytoplasm of mammary carcinoma cells occurs with a low degree of tumor differentiation do not differ from those of Won et al. (2021) because G3 tumor differentiation is associated with pronounced nuclear pleomorphism. In assessing RIPK3 expression in the tumor in

relation to the overall degree of differentiation of mammary carcinomas, Won et al. (2021) did not find a statistically significant relationship between G and RIPK3 expression, which differs from our results. Feng et al. (2015), similar to Won et al. (2021) also found no statistically significant difference between the cytoplasmic expression of RIPK3 in tumor cells and the degree of differentiation of colorectal cancer. In a study by Feng et al. (2015), as in our country, the majority of tumors with a low degree of differentiation show poor to no expression of RIPK3 in the cytoplasm, but probably due to the small number of G3 cases included in their study (only 11 out of 112) , the statistical analysis in their study did not show a significant difference compared with patients with G1 and G2 tumors. Although the study of Stefanova (2017) is dominated by colon carcinomas with G3 differentiation, which show a low degree of expression of RIPK3, she also did not report a statistically significant relationship between the two indicators (10 cases versus 4 cases of high expression). In contrast to previous studies, the results of the study by Karami-Tehrani et al. (2016) are fully in line with the data from the present study. The authors found that low levels of RIPK3 expression were found in low-grade mammary carcinomas in contrast to highly and moderately differentiated tumors, where expression was high. Like us, they did not find a significant relationship between RIPK3 expression levels between G1 and G2 carcinomas. Based on the data from the literature and those obtained by us, we can conclude that the discrepancies in the results obtained are most likely due to the insufficient number of cases included in the group of low-grade tumors.

Comparative analysis of cytoplasmic expression of RIPK3 depending on the extent of tumor spread (metastasis)

Correspondence analysis was used to assess the cytoplasmic expression of RIPK3 in relation to the extent of tumor spread. Table 12 shows the distribution of cases according to the presence or absence of metastases relative to the cytoplasmic expression of RIPK3 by percentiles. These data are presented graphically in Figure 31, which

shows that there is not a strong enough relationship between the studied indicators. This is also confirmed by the lack of a significant difference between the cytoplasmic expression of RIPK3 and the extent of tumor spread (p = 0.625).

Table 12. *Distribution of cases according to the degree of spread of tumors relative to cytoplasmic expression of RIPK3 by percentiles.*

Група	**RIPK3 (low expression)**	**RIPK3 (moderate expression)**	**RIPK3 (high expression)**	**P-value**
No metastases	12 (46,1%)	10 (40,0%)	9 (34,6%)	**0,625**
Metastases in regional LN	10 (38,5%)	11 (44,0%)	9 (34,6%)	
Distant metastases	4 (15,4%)	4 (16,0%)	8 (30,8%)	
Total	**26 (100%)**	**25 (100%)**	**26 (100%)**	

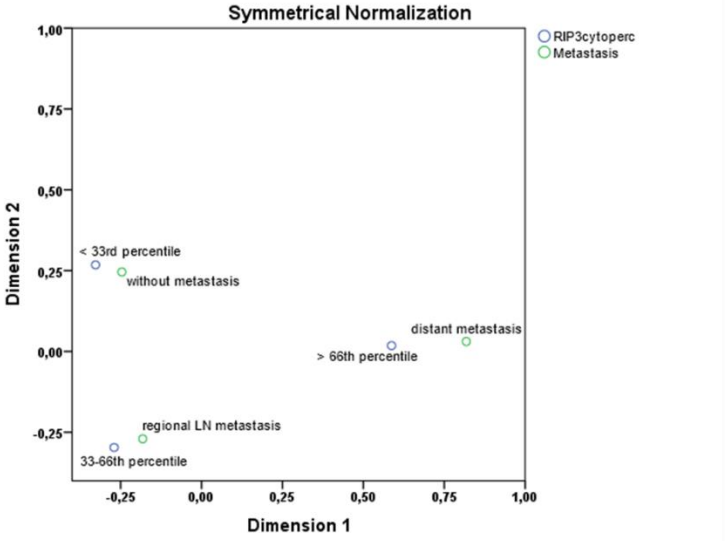


Figure 31. *Relationship between cytoplasmic RIPK3 expression and extent of tumor spread.*

Similar to the results obtained by us, Feng et al. (2015) and Stefanova (2017), analyzing the immunohistochemical cytoplasmic expression of RIPK3 in relation to the status of regional lymph nodes in colorectal carcinomas, did not establish a relationship between the two indicators.

Comparative analysis of cytoplasmic expression of RIPK3 depending on the molecular profile of the tumor

Comparative analysis of cytoplasmic expression of RIPK3 according to ER

Comparative analysis of cytoplasmic expression of RIPK3 depending on the area of ER expression in tumors (Proportion score - PS)

The strength of the relationship between cytoplasmic RIPK3 expression and the area of ER expression in tumors was assessed by Kendall's tau c. Its value of 0.197 shows a weak to moderately positive relationship between the studied indicators, which is associated with a tendency for a weak significant relationship between them (p = 0.079). The distribution of cases according to the prevalence of ER in tumors is shown in Table 13.

Table 13. *Distribution of cases according to the degree of prevalence of ER in tumors relative to the cytoplasmic expression of RIPK3 in the median.*

Group	**RIPK3 (low expression)**	**RIPK3 (high expression)**	**P-value**
ER PS0	18 (45,0%)	8 (20,5%)	**0,079**
ER PS2	0 (0,0%)	1 (2,6%)	
ER PS3	0 (0,0%)	1 (2,6%)	
ER PS4	2 (5,0%)	4 (10,3%)	
ER PS5	20 (50,0%)	25 (64,0%)	
Total	**40 (100%)**	**39 (100%)**	

Comparative analysis of cytoplasmic expression of RIPK3 depending on the intensity of ER expression in tumor cells (Intensity score - IS)

The strength of the relationship between cytoplasmic RIPK3 expression and the intensity of the ER response in tumor cells was determined by Kendall's tau c. Its value is 0.341, which shows a strong positive relationship between the studied indicators. This means that the cytoplasmic expression of RIPK3 and the intensity of the ER response in tumor cells are associated with a significant dependence ($p = 0.003$). With pronounced ER expression in tumor cell nuclei, cytoplasmic RIPK3 expression in cells is also high. Table 14 shows the distribution of cases according to the intensity of ER expression in tumors.

Table 14. *Distribution of cases according to the intensity of the ER response in tumors relative to the cytoplasmic expression of RIPK3 in the median.*

Group	RIPK3 (low expression)	RIPK3 (high expression)	P-value
ER IS0	18 (45,0%)	8 (20,5%)	**0,003**
ER IS1	2 (5,0%)	1 (2,6%)	
ER IS2	9 (22,5%)	8 (20,5%)	
ER IS3	11 (27,5%)	22 (56,4%)	
Total	**40 (100%)**	**39 (100%)**	

Comparative analysis of cytoplasmic expression of RIPK3 depending on the total score of ER in tumor cells (Total score - TS)

The Kendall's tau c coefficient was 0.297, indicating a moderate to strong positive relationship between cytoplasmic expression of RIPK3 and the total area and intensity of the ER response in tumor cells. This positive relationship is also supported by $p = 0.011$ values between the two indicators: cytoplasmic expression of RIPK3 and TS of ER in tumor cells. The results showed that the higher the TS of ER in tumor cells, the stronger the cytoplasmic response of RIPK3 in cells

(Figure 32). Conversely, the lower the TS of ER in tumor cells, the lower the cytoplasmic expression of RIPK3 in cells. Table 15 shows the distribution of cases according to the total score of the area and intensity of the reaction for ER and RIPK3 in tumor cells.

Table 15. *Distribution of cases according to ER TS in tumor cells versus cytoplasmic RIP3 expression by median.*

Group	**RIP3 (low expression)**	**RIP3 (high expression)**	**P-value**
ER TS0	18 (45,0%)	8 (20,5%)	**0,011**
ER TS4	0 (0,0%)	1 (2,6%)	
ER TS5	1 (2,5%)	2 (5,1%)	
ER TS6	2 (5,0%)	1 (2,6%)	
ER TS7	8 (20,0%)	7 (17.9%)	
ER TS8	11 (27,5%)	20 (51,3%)	
Total	**40 (100%)**	**39 (100%)**	

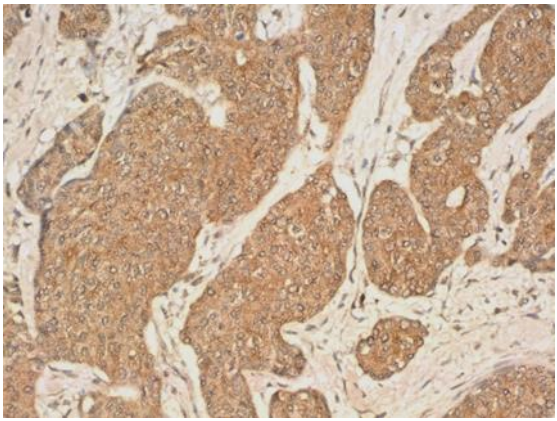


Figure 32. *High cytoplasmic expression of RIPK3 in ER-positive tumor with TS = 8, x 200.*

The results obtained by us are in accordance with the data of the study of Karami-Tehrani et al. (2016), who reported a significant relationship between RIPK3 expression in tumor cells and ER status of mammary carcinomas. They found that ER-negative tumors were

associated with lower levels of necroptosis marker expression. Not all authors find such a dependence. According to Won et al. (2021) there is no relationship between the expression of RIPK3 and ER.

Comparative analysis of PR-dependent cytoplasmic expression of RIPK3

Comparative analysis of cytoplasmic expression of RIPK3 depending on the area of PR expression in tumors (Proportion score - PS)

The value of the Kendall's tau c coefficient for the relationship between cytoplasmic RIPK3 expression and the prevalence of PR in tumors is 0.322. It shows the presence of a strong positive relationship between the two indicators, which is confirmed by the established significant relationship between them ($p = 0.005$). These data suggest that the more progesterone tumor cells express, the more pronounced the cytoplasmic response to RIPK3. The distribution of cases according to the prevalence of PR in tumors is shown in Table 16.

Table 16. *Distribution of cases according to the degree of prevalence of PR in tumors relative to the cytoplasmic expression of RIPK3 in the median.*

Group	**RIPK3 (low expression)**	**RIPK3 (high expression)**	**P-value**
PR PS0	23 (57,5%)	12 (30,8%)	**0,005**
PR PS1	1 (2,5%)	1 (2,6%)	
PR PS2	3 (7,5%)	2 (5,1%)	
PR PS3	2 (5,0%)	4 (10,3%)	
PR PS4	5 (12,5%)	6 (15,4%)	
PR PS5	6 (15,0%)	14 (35,9%)	
Total	**40 (100%)**	**39 (100%)**	

Comparative analysis of cytoplasmic expression of RIPK3 depending on the intensity of PR expression in tumor cells (Intensity score - IS)

The relationship between the cytoplasmic expression of RIPK3 and the intensity of the PR response in tumor cells based on the Kendall's tau c coefficient is 0.334, which means a strong positive relationship between them. These results are associated with the presence of a significant relationship between cytoplasmic expression of RIPK3 and the intensity of the PR response in tumor cells ($p = 0.003$). The data show that the more pronounced the expression of progesterone in the nuclei of tumor cells, the stronger the cytoplasmic response of RIPK3 in cells. Table 17 shows the distribution of cases according to the two stages of PR expression in tumors.

Table 17. *Distribution of cases according to the intensity of the PR response in tumors relative to the median cytoplasmic expression of RIPK3.*

Group	RIPK3 (low expression)	RIPK3 (high expression)	P-value
PR IS0	23 (57,5%)	12 (30,8%)	**0,003**
PR IS1	4 (10,0%)	3 (7,7%)	
PR IS2	9 (22,5%)	13 (33,3%)	
PR IS3	4 (10,0%)	11 (28,2%)	
Total	**40 (100%)**	**39 (100%)**	

Comparative analysis of cytoplasmic expression of RIPK3 depending on the total rate of spread and intensity of the reaction for PR in tumor cells (Total score - TS)

The Kendall's tau c coefficient for the relationship between the two indicators: cytoplasmic expression of RIPK3 and the total rate of spread and intensity of the PR response in tumor cells is 0.333, which means a strong positive relationship between the two indicators. This positive relationship is also supported by $p = 0.004$ values, indicating the presence of a significant relationship between cytoplasmic expression of RIPK3 and TS of PR in tumor cells. At high TS of PR

in tumor cells, cytoplasmic expression of RIPK3 in cells is also high. Table 18 shows the distribution of cases according to the total rate of spread and intensity of the PR response in tumor cells.

Table 18. *Distribution of cases according to TS of PR in tumor cells versus cytoplasmic expression of RIPK3 by median.*

Group	**RIPK3 (low expression)**	**RIPK3 (low expression)**	**P-value**
PR TS0	23 (57,5%)	12 (30,8%)	**0,004**
PR TS2	0 (0,0%)	1 (2,6%)	
PR TS3	3 (7,5%)	0 (0,0%)	
PR TS4	1 (2,5%)	1 (2,6%)	
PR TS5	3 (7,5%)	7 (17,9%)	
PR TS6	5 (12,5%)	2 (5,1%)	
PR TS7	1 (2,5%)	8 (20,5%)	
PR TS8	4 (10,0%)	8 (20,5%)	
Total	**40 (100%)**	**39 (100%)**	

The results presented in the present study differ from the data obtained by Karami-Tehrani et al. (2016) and by Won et al. (2021). In these two studies, no statistically significant relationship between the level of RIPK3 expression in tumor cells and the PR status of carcinomas was found.

Comparative analysis of cytoplasmic expression of RIPK3 depending on HER2 expression

The cytoplasmic expression of RIPK3 in neoplastic tissue in relation to the expression of HER2 by tumor cells was initially analyzed by a χ^2 assay that found no relationship between the two parameters ($p = 0.577$) (Table 19).

Table 19. *Distribution of tumors by HER2 status relative to cytoplasmic RIPK3 expression by median.*

Group	**RIPK3 (low expression)**	**RIPK3 (high expression)**	**P-value**
HER2-positive	12 (30,0%)	14 (35,9%)	**0,577**
HER2-negative	28 (70,0%)	25 (64,1%)	
Total	**40 (100%)**	**39 (100%)**	

Additional analysis of data from the Mann-Whitney test to assess the relationship between cytoplasmic RIPK3 expression and HER2 expression by tumor cells also showed that it was absent (p = 0.342). The mean values of cytoplasmic expression of the necroptosis marker in the group of HER2-positive tumors are relatively close to those of HER2-negative carcinomas, with values of 131.5 ± 45.27 and 113.8 ± 60.79, respectively (Table 20).

Table 20. *Relationship between cytoplasmic RIPK3 expression and HER2 expression.*

Group	Cases (n)	Mean cytoplastimc expression of RIPK3	Standard deviation	P-value
HER2(+)	26	131,5	45,27	**0,342**
HER2(-)	53	113,8	60,79	

These results are consistent with data obtained from Karami-Tehrani et al. (2016), who, like us, did not find a relationship between the level of expression of RIPK3 and that of HER2 in tumor cells. The data obtained from us differ from those of Won et al. (2021), who reported a statistically significant inverse relationship between the cytoplasmic level of RIPK3 in tumor cells and the HER2 status of the tumor. According to the authors, HER2-positive carcinomas have a low level of immunohistochemical cytoplasmic expression of RIPK3 in tumor cells.

Comparative analysis of cytoplasmic expression of RIPK3 in triple-negative tumors compared to all other carcinomas

Chi-square analysis showed a statistically significant relationship between the level of cytoplasmic expression of RIPK3 in triple-negative tumors on the one hand and all other carcinomas included in the study on the other (p = 0.026) (Table 21).

Table 21. *Distribution of cases in the individual groups of tumors in relation to the cytoplasmic expression of RIPK3 by the median.*

Group	**RIPK3 (low expression)**	**RIPK3 (high expression)**	**P-value**
Triple negative tumors	15 (37,5%)	6 (15,4%)	**0,026**
ER(+), PR(+) or PR(-) and HER2(+) tumors	25 (62,5%)	33 (84,6%)	
Total	**40 (100%)**	**39 (100%)**	

Analyzing the mean values of cytoplasmic expression of RIPK3 in triple-negative tumors on the one hand and all other cases (ER-positive, PR-positive or PR-negative and HER2-positive) on the other hand confirmed the statistically significant difference in marker values for necroptosis between the two groups of tumors ($p = 0.002$). The mean cytoplasmic expression of RIPK3 in all 21 cases of triple-negative carcinomas was 80.5 ± 60.85 (Figure 33), which was significantly lower than the other 58 patients with ER or HER2-positive tumors, for which the values are 133.8 ± 47.94 (Table 22).

Table 22. *Relationship between mean cytoplasmic RIPK3 expression in triple-negative tumors compared to all other carcinomas studied.*

Group	**Cases (n)**	**Mean cytoplastic expression of RIPK3**	**Standard deviation**	**P-value**
Triple negative tumors	21	80,5	60,85	**0,002**
ER(+), PR(+) or PR(-) and HER2(+) tumors	58	133,8	47,94	

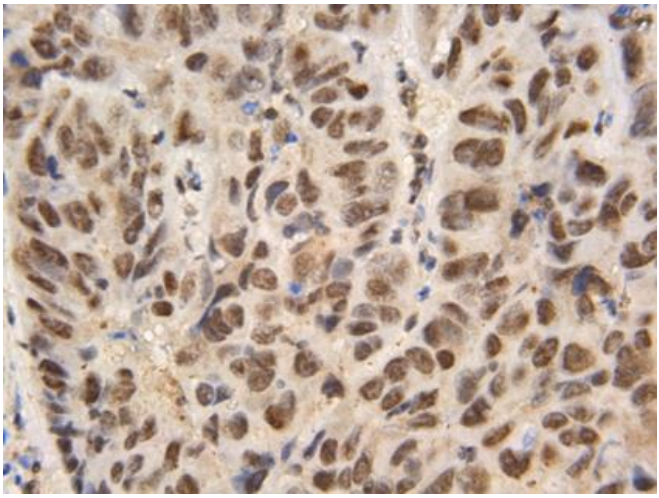


Figure 33. *Low cytoplasmic and high nuclear expression of RIPK3 in triple-negative tumors, x400.*

We compared the data we obtained with those of Won et al. (2021), who, like us, analyzed the immunohistochemical cytoplasmic expression of RIPK3 in triple-negative carcinomas versus positive for ER, PR, or HER2 tumors, but unlike us, they did not find a statistically significant difference between the groups. In the triple-negative cancers included in their study, 28 (25.2%) cases had high levels of RIPK3 expression, while 42 (28.8%) cases had low positivity compared to 6 (15.4%) cases with high expression in the present study. study and 15 (37.5%) with low positivity. Although Won et al. (2021) did not find a statistically significant difference, the expression pattern is similar. Low expression is more common in triple-negative cancers than high expression.

Comparative analysis of cytoplasmic expression of RIPK3 depending on Ki67 proliferative activity index

The Chi-square test showed a statistically significant relationship between cytoplasmic expression of RIPK3 and proliferative activity as determined by Ki67 ($p = 0.046$) (Table 23).

Table 23. *Distribution of Ki67 tumors relative to median cytoplasmic RIPK3 expression.*

Group	**RIPK3 (low expression)**	**RIPK3 (high expression)**	**P-value**
Ki67<14%	9 (22,5%)	17 (43,6%)	**0,046**
Ki67≥14%	31 (77,5%)	22 (56,4%)	
Total	**40 (100%)**	**39 (100%)**	

Table 24 shows the data from the analysis between the cytoplasmic expression of RIPK3 and Ki67 by the Mann-Whitney test. The mean cytoplasmic response for RIPK3 in tumors with low and high proliferative activity was 144.0 ± 48.27 and 107.6 ± 56.76, respectively. From the data it is clear that there is a statistically significant difference between the values of RIPK3 in the two groups of tumors ($p = 0.015$). The results show that low Ki67 expression in tumor cells has a high cytoplasmic response to RIPK3 in cells and vice versa.

Table 24. *Relationship between cytoplasmic expression of RIPK3 and Ki67.*

Group	**Cases (n)**	**Mean cytoplastimc expression of RIPK3**	**Standard deviation**	**P-value**
Ki67<14%	26	144,0	48,27	**0,015**
Ki67≥14%	53	107,6	56,76	

The results obtained by us differ from the data of Karami-Tehrani et al. (2016) and Won et al. (2021), who found no statistically significant relationship between RIPK3 expression and the proliferative marker Ki67 in tumor cells.

Comparative analysis of Disease-free survival (DFS) versus cytoplasmic RIPK3 expression in tumors

Survival data from the cases we analyzed were available for 58 patients. The cytoplasmic expression of RIPK3 in the tumors of these patients was distributed by median, with 30 cases being found to be

low in expression and the remaining 28 cases to be high in cytoplasmic response.

To investigate the effect of cytoplasmic RIPK3 expression in tumor tissue on DFS, we used the Kaplan-Meier test. The average survival in months is 117.9 months. (CI, 104.2 - 131.7) for cases with low cytoplasmic expression of RIPK3 and 107.9 months. (CI, 89.9 - 125.9) for tumors with a high cytoplasmic response. We did not find a statistically significant difference in patient survival depending on the cytoplasmic expression of RIPK3 in tumor tissue (log rank p = 0.36) (Fig. 34).

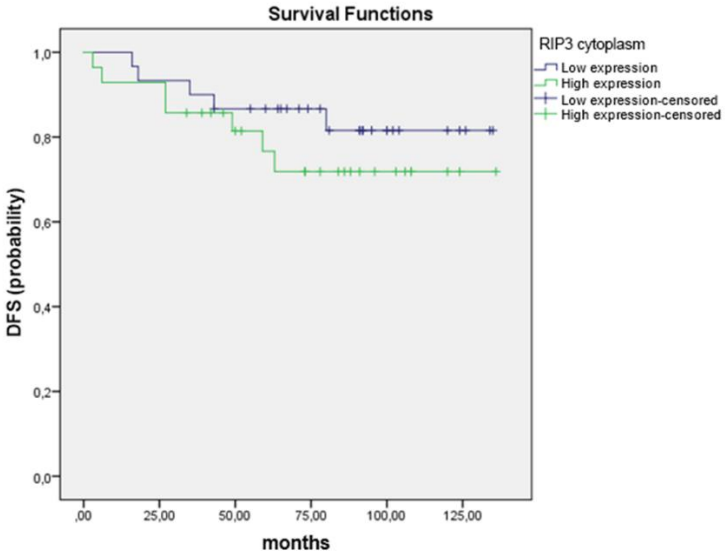


Figure 34. *Kaplan-Meier curves of disease-free survival distribution in patients with mammary carcinoma depending on the cytoplasmic expression of RIPK3 in tumor tissue.*

The obtained results are in accordance with the data obtained by Lomphithak et al. (2021), who studied 88 cases of cholangiocellular carcinoma and found no significant relationship between immunohistochemical cytoplasmic expression of RIPK3 in tumor cells and the survival of patients without disease.

Comparative analysis of nuclear expression of RIPK3 depending on clinical and morphological characteristics, molecular profile, proliferative marker Ki67 and survival of patients with breast cancer

Comparative analysis of nuclear expression of RIPK3 depending on the clinical and morphological characteristics of the tumor

Comparative analysis of nuclear expression of RIPK3 depending on the age of the patients

The Chi-square test for the analysis of low and high nuclear expression of RIPK3 in tumor tissue in relation to the age of patients showed that there was no statistically significant relationship between the nuclear response of the necroptotic marker in patients under and over 65 years of age (p = 0.528) (Table 25).

Table 25. *Distribution of patients by age versus median RIPK3 expression by median.*

Group	**RIPK3 (low expression)**	**RIPK3 (high expression)**	**P-value**
≤ 65 y	25 (62,5%)	27 (69,2%)	**0,528**
> 65 y	15 (37,5%)	12 (30,8%)	
Total	**40 (100%)**	**39 (100%)**	

The Mann-Whitney analysis also did not show a statistical relationship between the mean values of RIPK3 nuclear expression and the age of the patients (p = 679). The mean value of nuclear expression of RIPK3 in the group of patients under 65 years. age was 190.5 ± 56.63, and in the remaining 27 patients over 65 it was almost the same - 187.2 ± 50.18.9 (Table 26).

Table 26. *Relationship between nuclear expression of RIPK3 and histological variant of the tumor.*

Group	Cases (n)	Mean nuclear expression of RIPK3	Standard deviation	P-value
≤ 65 y	52	190,5	56,63	**0,679**
> 65 y	27	187,2	50,18	

Our results for the lack of relationship between nuclear expression of RIPK3 and the age of patients do not differ from those of Stefanova (2017), who divides the nuclear expression of RIPK3 in tumor cells into low and high according to the cut-off value. In her study of colorectal cancer, the cases were also divided into two groups, ≤ 65 years and> 65 years, and similarly to the present study, no statistically significant difference was found between the levels of RIPK3 nuclear expression depending on the age of the patients.

Comparative analysis of nuclear expression of RIPK3 depending on the histological type of the tumor

The χ^2 test was used to analyze the nuclear expression of RIPK3 in relation to the histological type of the tumor. Significant dependence between the two indicators was not established (p = 0.239) (Table 27).

Table 27. *Distribution of tumors by histological variant versus median RIPK3 nuclear expression.*

Group	RIPK3 (low expression)	RIPK3 (high expression)	P-value
Infiltrating duct carcinoma (NOS)	34 (85%)	29 (74,4%)	**0,239**
Lobular carcinoma	6 (15%)	10 (25,6%)	
Total	**40 (100%)**	**39 (100%)**	

The comparative analysis of the data between the nuclear expression of RIPK3 and the histological variant of the tumor by the

Mann-Whitney test also did not show a statistically significant relationship between the two studied indicators (p = 0.683). The mean values of nuclear expression of the marker for necroptosis are similar in both types of tumors - in ductal carcinoma it is 190.4 ± 51.37, and in lobular carcinoma 185.3 ± 65.97, which explains the lack of significant relationship between the analyzed indicators (Table 28).

Table 28. *Comparative analysis between nuclear expression of RIPK3 and histological variant of the tumor.*

Group	**Cases (n)**	**Mean nuclear expression of RIPK3**	**Standard deviation**	**P-value**
Infiltrating duct carcinoma (NOS)	63	190,4	51,37	**0,683**
Lobular carcinoma	16	185,3	65,97	

Comparative analysis of nuclear expression of RIPK3 depending on the T-stage of tumors

The comparative analysis between the nuclear expression of RIPK3 and the T-stage of the studied tumors was initially checked by χ^2 test, which did not show a statistically significant relationship between the two studied indicators (p = 0.249) (Table 29).

Table 29. *Distribution of T-stage cases relative to the low and high nuclear expression of RIPK3 in the median.*

Group	**RIPK3 (low expression)**	**RIPK3 (high expression)**	**P-value**
T1	17 (55,0%)	16 (51,3%)	**0,249**
T2	22 (42,5%)	20 (41,0%)	
T3	1 (2,5%)	0 (0,0%)	
T4	0 (0,0%)	3 (7,7%)	
Total	**40 (100%)**	**39 (100%)**	

Comparative analysis of the data by the Mann-Whitney test between the mean values of nuclear expression of RIPK3 and T-stage of tumors was performed only for cases diagnosed in T1 and T2 stage.

The determined p = 0.477 shows no statistically significant relationship between the two indicators. Tumors in T1 stage were 33 cases with mean expression values of the necroptotic nuclear marker 182.8 ± 47.91, and tumors in T2 stage were 42 cases with mean values of 193.3 ± 58.31 (Table 30). For the three T4 stage tumors, mean nuclear expression values were determined to be 235.0 ± 21.79. In stage T3 there is only one tumor. Due to the small number of cases in the latter two categories, they were not included in the Mann-Whitney test (Figure 35).

Table 30. *Relationship between nuclear expression of RIPK3 and T-stage of tumors.*

Group	Cases (n)	Mean nuclear expression of RIPK3	Standard deviation	P-value
T1	33	182,8	47,91	**0,477**
T2	42	193,3	58,31	

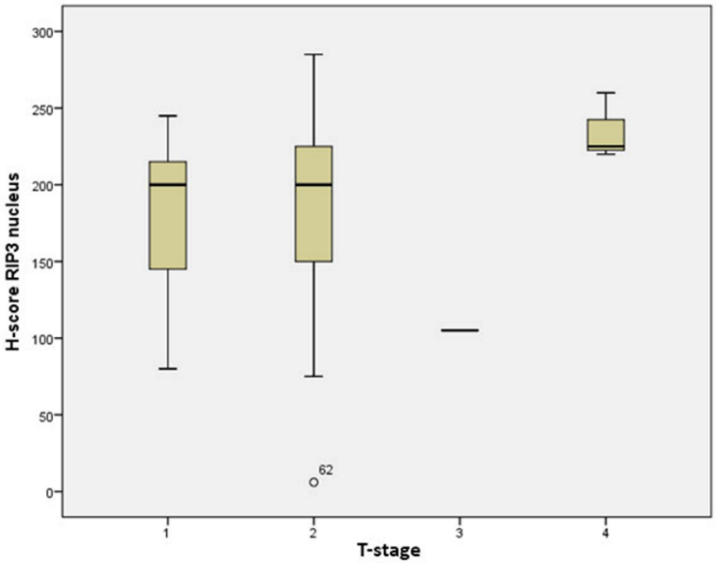


Figure 35. *Distribution of T-stage cases compared to the mean values of nuclear expression of RIPK3 in tumor cells.*

The results presented by us correspond to those in the study of Stefanova (2017), who also did not find a statistically significant difference between the level of nuclear expression of RIPK3 in colorectal cancer depending on the T-stage of the disease.

Comparative analysis of the mean values of nuclear expression of RIPK3 depending on the degree of tumor differentiation (G)

A comparative analysis, by the Kruskal-Wallis test, of the mean values of nuclear expression of RIPK3 in tumor cells in relation to the degree of differentiation of carcinomas did not show a statistically significant difference ($p = 0.896$) (Figure 36). The mean values of nuclear expression of RIPK3 in tumors with high, moderate and low degree of differentiation were 185.6 ± 63.88, 189.6 ± 47.95 and 191.4 ± 56.72, respectively, and are presented in Table 31.

Table 31. *Comparative analysis between the mean values of nuclear expression of RIPK3 and the degree of tumor differentiation.*

Group	Cases (n)	Mean nuclear expression of RIPK3	Standard deviation	P-value
G1	17	185,6	63,88	**0,896**
G2	33	189,6	47,95	
G3	29	191,4	56,72	

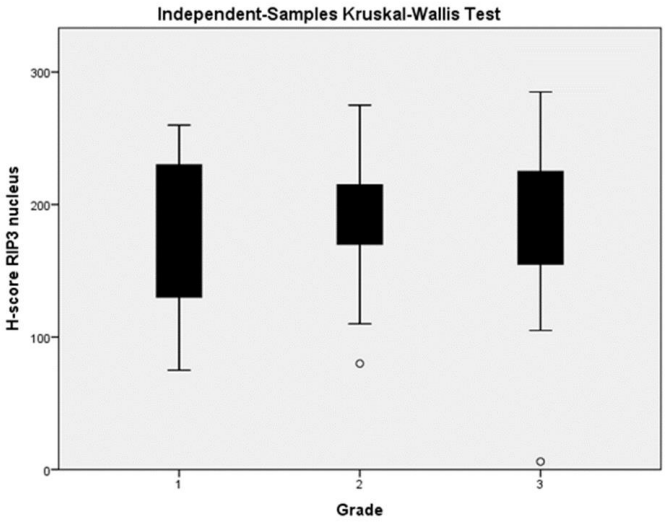


Figure 36. *Distribution of breast carcinomas according to their degree of differentiation relative to the mean values of nuclear expression of RIPK3 in tumor cells.*

Additional analysis of the data between the different groups of tumors with the three different degrees of differentiation was performed and the groups were compared in pairs (Pairwise Comparisons). This analysis also did not show a statistically significant difference between the indicators. Examination of RIPK3 nuclear expression values in the G1 and G2 groups showed $p = 0.721$, in the G2 and G3 tumor pair $p = 0.836$, and in the comparison of RIPK3 nuclear expression in G1 and G3 tumors $p = 0.667$.

The data from the present study correspond to the results published by Stefanova (2017), which also did not show a significant relationship between nuclear expression of RIPK3 in tumor cells and the degree of differentiation of colorectal cancer.

Comparative analysis of nuclear expression of RIPK3 to stage N and M

Correspondence analysis compared data from the nuclear expression of RIPK3 in relation to the extent of tumor spread. Table 32 shows the distribution of cases according to the presence or absence of lymphatic and distant metastases relative to the nuclear expression of RIPK3 by percentiles. Figure 37 shows the relationship between the nuclear expression of the necroptosis marker and the extent of tumor spread. There is a significant relationship between RIPK3 nuclear expression and regional lymph node metastases ($p = 0.049$). The higher the expression of RIPK3 in the nuclei of tumor cells, the more likely the tumor is to have lymph node metastases. The other two groups (no metastases and distant metastases) had no statistically significant dependence on nuclear expression of RIPK3.

Table 32. *Distribution of cases according to the extent of tumor spread relative to nuclear expression of RIPK3 by percentiles.*

Група	**RIPK3 (low expression)**	**RIPK3 (moderate expression)**	**RIPK3 (high expression)**	**P-value**
No metastases	16 (64,0%)	7 (25,9%)	8 (32,0%)	
Metastases in regional LN	7 (28,0%)	12 (29,6%)	11 (44,0%)	**0,049**
Distant metastases	2 (8,0%)	8 (44,5%)	6 (24,0%)	
Общо	**25 (100%)**	**27 (100%)**	**25 (100%)**	

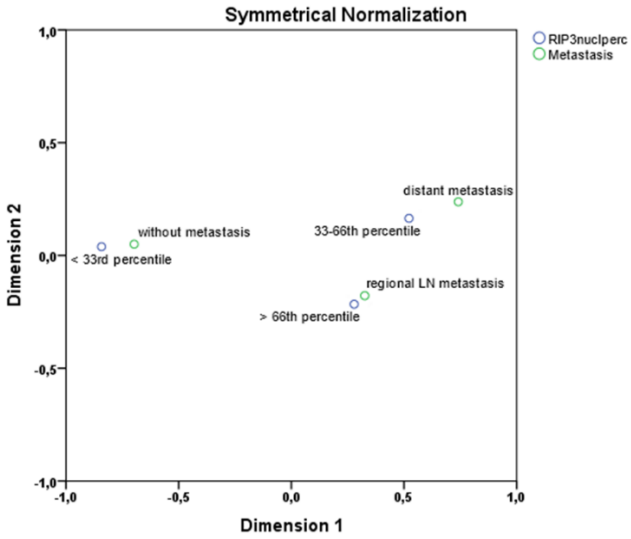


Figure 37. *Relationship between nuclear expression of RIPK3 and tumor prevalence.*

There are no data in the literature on the relationship between nuclear expression of RIPK3 and the prevalence of breast cancer. There are studies that analyze the relationship between the cytoplasmic expression of the marker of necroptosis in tumor cells and the likelihood of tumor metastasis. Won et al. (2021) reported that low cytoplasmic expression of RIPK3 was more likely to metastasize to regional lymph nodes in patients with breast cancer than high expression. Weber et al. (2018) suggest that the retention of RIPK3 and MLKL in the nucleus prevents the process of cytosolic RIPK3 / MLKL oligomerization and, as a result, blocks cell death. In our opinion, high nuclear expression of RIPK3 can be perceived as a way for tumor cells to escape necroptosis by retaining RIPK3 in the cell nucleus. The mechanisms involved in this process are still unclear. Taken together, the literature data and our results indicate that low cytoplasmic expression and high nuclear expression of RIPK3 are associated with an increased risk of regional lymph node metastases.

In colorectal cancer, a significant relationship between the nuclear expression of RIPK3 in tumor cells and the N-stage of colon cancer has not been established (Stefanova, 2017).

Comparative analysis of RIPK3 nuclear expression depending on the molecular profile of the tumor

Comparative analysis of ER-dependent nuclear expression of RIPK3

Comparative analysis of RIPK3 nuclear expression depending on the area of ER expression in tumors (Proportion score - PS)

The comparative analysis between the nuclear expression of RIPK3 in relation to the area of ER expression in tumors was performed with the Kendall's tau c coefficient. Its value of 0.148 shows a weak positive relationship between the studied indicators, but there is no significant relationship between them (p = 0.195). The distribution of cases according to the area of ER prevalence in tumors is shown in Table 33.

Table 33. *Distribution of cases according to the area of ER expression in tumors relative to the nuclear expression of RIPK3 in the median.*

Group	RIPK3 (low expression)	RIPK3 (high expression)	P-value
ER PS0	14 (35,0%)	12 (30,8%)	**0,195**
ER PS2	1 (2,5%)	0 (0,0%)	
ER PS3	1 (2,5%)	0 (0,0%)	
ER PS4	5 (12,5%)	1 (2,6%)	
ER PS5	19 (47,5%)	26 (66,7%)	
Total	**40 (100%)**	**39 (100%)**	

Comparative analysis of nuclear expression of RIPK3 in relation to the intensity of ER expression in tumor cells (Intensity score - IS)

The comparative analysis between the nuclear expression of RIPK3 in relation to the intensity of nuclear expression of ER in tumor cells was performed using the Kendall's tau c coefficient. The obtained value of 0.151 shows a weak positive relationship between the indicators: nuclear expression of RIPK3 and the intensity of the reaction for ER, but the relationship is not significant (p = 0.213). Table 34 shows the distribution of cases according to the intensity of ER expression in tumors.

Table 34. *Distribution of cases according to the intensity of the ER response in tumors relative to the nuclear expression of RIPK3 in the median.*

Group	**RIPK3 (low expression)**	**RIPK3 (high expression)**	**P-value**
ER IS0	14 (35,0%)	12 (30,8%)	**0,213**
ER IS1	2 (5,0%)	1 (2,6%)	
ER IS2	11 (27,5%)	6 (15,4%)	
ER IS3	13 (32,5%)	20 (51,3%)	
Total	**40 (100%)**	**39 (100%)**	

Comparative analysis of nuclear expression of RIPK3 depending on the total score (area and intensity of the ER response in tumor cells) (Total score - TS)

The Kendall's tau c coefficient was used to analyze the nuclear expression of RIPK3 in relation to the total score of the area of distribution and intensity of the ER response in tumor cells. The obtained value of 0.176 shows a weak positive relationship between the two indicators, but it is not significant (p = 0.150). Table 35 shows the distribution of cases according to the total ER score and nuclear expression of RIPK3 in tumor cells.

Table 35. *Distribution of cases according to ER TS in tumor cells versus nuclear RIPK3 expression by median.*

Group	RIPK3 (low expression)	RIPK3 (high expression)	P-value
ER TS0	14 (35,0%)	12 (30,8%)	**0,150**
ER TS4	1 (2,5%)	0 (0,0%)	
ER TS5	3 (7,5%)	0 (0,0%)	
ER TS6	2 (5,0%)	1 (2,6%)	
ER TS7	8 (20,0%)	7 (17,9%)	
ER TS8	12 (30,0%)	19 (48,7%)	
Total	**40 (100%)**	**39 (100%)**	

Comparative analysis of PR-dependent nuclear expression of RIPK3

Comparative analysis of RIPK3 nuclear expression depending on the area of PR expression in tumors (Proportion score - PS)

The Kendall's tau c coefficient was used to analyze the nuclear expression of RIPK3 in relation to the area of PR expression in tumors. Its value of -0.185 indicates the presence of a weak negative relationship between the two indicators, but without finding a significant relationship between them (p = 0.131). The distribution of cases according to the area of PR expression in tumors is shown in Table 36.

Table 36. *Distribution of cases according to the area of PR expression in tumors relative to the nuclear expression of RIPK3 in the median.*

Group	RIPK3 (low expression)	RIPK3 (high expression)	P-value
PR PS0	17 (42,5%)	18 (46,2%)	**0,131**
PR PS1	0 (0,0%)	2 (5,1%)	
PR PS2	2 (5,0%)	3 (7,7%)	
PR PS3	1 (2,5%)	5 (12,8%)	
PR PS4	5 (12,5%)	6 (15,4%)	
PR PS5	15 (37,5%)	5 (12,8%)	
Total	**40 (100%)**	**39 (100%)**	

Comparative analysis of nuclear expression of RIPK3 depending on the intensity of PR expression in tumor cells (Intensity score - IS)

The comparative analysis of nuclear expression of RIPK3 in relation to the intensity of the PR response in tumor cells was performed using the Kendall's tau c coefficient. The obtained value of -0.095 shows a very weak negative relationship between the studied indicators. The value is very close to 0, which means almost no dependence between the nuclear expression of RIPK3 and the intensity of the PR response in tumor cells, which is confirmed by the value of p = 0.436. Table 37 shows the distribution of cases according to the intensity of PR expression in tumors.

Table 37. *Distribution of cases according to the intensity of the PR response in tumors relative to the nuclear expression of RIPK3 in the median.*

Group	**RIPK3 (low expression)**	**RIPK3 (high expression)**	**P-value**
PR IS0	17 (42,5%)	18 (46,2%)	**0,436**
PR IS1	2 (5,0%)	5 (12,8%)	
PR IS2	12 (30,0%)	10 (25,6%)	
PR IS3	9 (22,5%)	6 (15,4%)	
Total	**40 (100%)**	**39 (100%)**	

Comparative analysis of nuclear expression of RIPK3 depending on the total area score and response intensity for PR in tumor cells (Total score - TS)

By determining the Kendall's tau c coefficient, the nuclear expression of RIPK3 was analyzed in relation to the total score of area and intensity of the PR response in tumor cells. The obtained value of -0.160 showed a weak negative relationship between the studied parameters, but the relationship between the nuclear expression of RIPK3 and TS of PR in tumor cells is not significant (p = 0.197). Table 38 shows the distribution of cases according to the total rate of spread and intensity of the PR response in tumor cells.

Table 38. *Distribution of cases according to PR of PR in tumor cells versus nuclear expression of RIPK3 by median.*

Group	RIPK3 (low expression)	RIPK3 (high expression)	P-value
PR TS0	17 (42,5%)	18 (46,2%)	**0,197**
PR TS2	0 (0,0%)	1 (2,6%)	
PR TS3	0 (0,0%)	3 (7,7%)	
PR TS4	1 (2,5%)	1 (2,6%)	
PR TS5	3 (7,5%)	7 (17,9%)	
PR TS6	4 (10,0%)	3 (7,7%)	
PR TS7	8 (20,0%)	1 (2,6%)	
PR TS8	7 (17,5%)	5 (12,8%)	
Total	**40 (100%)**	**39 (100%)**	

Comparative analysis of RIPK3 nuclear expression in relation to HER2 expression in tumors

Nuclear expression of RIPK3 in relation to HER2 status of tumors was analyzed by the Chi-square test, but no statistically significant relationship between the nuclear marker for necroptosis in the two groups of tumors - HER2-positive and HER2-negative - was found (p = 0.689) (Table 39).

Table 39. *Dependence of nuclear expression of RIPK3 in relation to HER2 expression.*

Group	RIPK3 (low expression)	RIPK3 (high expression)	P-value
HER2-positive	14 (35,0%)	12 (30,8%)	**0,689**
HER2-negative	26 (65,0%)	27 (69,2%)	
Total	**40 (100%)**	**39 (100%)**	

Table 40 shows the nuclear expression of RIPK3 in relation to HER2 expression in different tumors. The mean values of the RIPK3 response in the nuclei of tumor cells in the 26 cases of HER2-positive carcinomas were 187.3 ± 48.40, and in the group of 53 HER2-negative tumors were 190.5 ± 57.26. There was no statistically significant

difference between the nuclear values of RIPK3 in the two groups of tumors (p = 0.484).

Table 40. *Comparative analysis between nuclear expression of RIPK3 and HER2 expression*

Group	Cases (n)	Mean nuclear expression of RIPK3	Standard deviation	P-value
HER2 (+)	26	187,3	48,40	**0,484**
HER2 (-)	53	190,5	57,26	

Comparative analysis between nuclear expression of RIPK3 in triple-negative tumors compared to all other carcinomas

The χ^2 test was used to analyze the nuclear expression of RIPK3 in triple-negative carcinomas relative to all other tumors - ER-positive, PR-positive or PR-negative and HER2-positive. No statistically significant difference was found between the two groups (p = 0.852) (Table 41).

Table 41. *Distribution of cases in the individual groups of tumors in relation to the nuclear expression of RIPK3 by the median.*

Group	RIPK3 (low expression)	RIPK3 (high expression)	P-value
Triple negative tumors	11 (27,5%)	10 (25,6%)	**0,852**
ER(+), PR(+) or PR(-) and HER2(+) tumors	29 (72,5%)	29 (74,4%)	
Total	**40 (100%)**	**39 (100%)**	

The Mann-Whitney test was used to analyze the nuclear expression of RIPK3 in triple-negative tumors on the one hand and all other carcinomas (ER-positive, PR-positive or PR-negative or HER2-positive) on the other. The mean values of nuclear expression in all 21 cases of triple-negative tumors was 195.1 ± 61.19, while in the remaining 58 tumors they were 187.3 ± 51.88. The expression of

RIPK3 in the two groups did not show a statistically significant difference (p = 0.474) (Table 42).

Table 42. *Relationship between nuclear expression of RIPK3 and triple-negative tumors compared to all other cancers studied.*

Group	**Cases (n)**	**Mean nuclear expression of RIPK3**	**Standard deviation**	**P-value**
Triple negative tumors	21	195,1	61,19	**0,474**
ER(+), PR(+) or PR(-) and HER2(+) tumors	58	187,3	51,88	

Comparative analysis of RIPK3 nuclear expression in relation to Ki67 proliferative activity index

The evaluation of nuclear expression of RIPK3 in relation to the Ki67 value in tumors was initially performed by χ^2 test, which did not find a relationship between the two indicators (p = 0.379) (Table 43).

Table 43. *Distribution of tumors according to Ki67 relative to the nuclear median expression of RIPK3.*

Group	**RIPK3 (low expression)**	**RIPK3 (high expression)**	**P-value**
Ki67<14%	15 (37,5%)	11 (28,2%)	**0,379**
Ki67≥14%	25 (62,5%)	28 (71,8%)	
Total	**40 (100%)**	**39 (100%)**	

Analysis by the Mann-Whitney test of the mean values of nuclear expression of RIPK3 in relation to the degree of proliferative activity of tumors also did not reveal a statistically significant difference (p = 0.125). The mean values of nuclear expression in the 26 cases with Ki67 <14% were 174.3 ± 57.69, while in the remaining 53 tumors with Ki67≥14% were 196.8 ± 51.36 (Table 44).

Table 44. *Relationship between nuclear expression of RIPK3 in tumors and Ki67.*

Group	Cases (n)	Mean nuclear expression of RIPK3	Standard deviation	P-value
Ki67<14%	26	174,3	57,69	**0,125**
Ki67≥14%	53	196,8	51,36	

Comparative analysis of Disease-free survival (DFS) versus nuclear expression of RIPK3 in tumors

Nuclear RIPK3 expression in tumors with no disease-free survival was distributed by median and was found to be low in 31 cases and high in the remaining 27 cases. The Kaplan-Meier test was used to assess the nuclear expression of RIPK3 in tumor tissue in relation to disease-free survival. The average survival of patients in months is 126.0 months. (CI, 115.2 - 136.7) for cases of low nuclear expression of RIPK3 and 99.9 months. (CI, 80.8 - 119.1) for those with high expression. The difference in progression-free survival in patients with low and high nuclear expression of RIPK3 was statistically significant (log rank p = 0.033) (Figure 38). With high nuclear expression of RIPK3 in tumor cells, survival is lower compared with patients with low levels of nuclear expression of RIPK3.

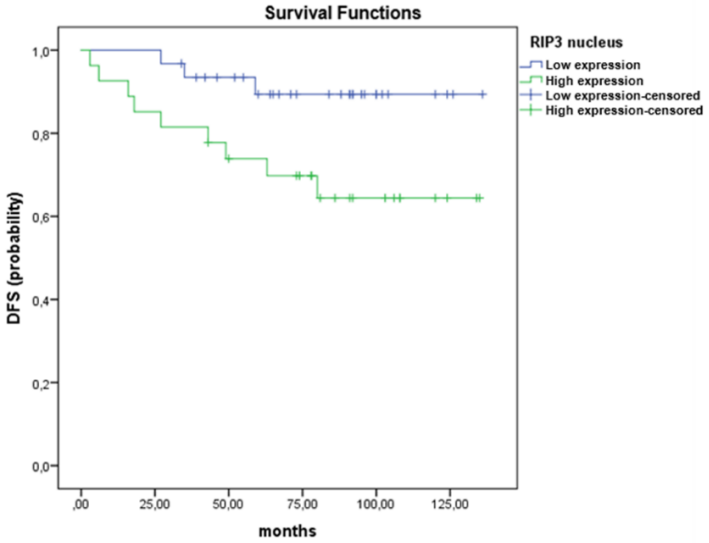


Figure 38. *Kaplan-Meier disease-free survival distribution curves in mammary carcinoma patients based on nuclear expression of RIPK3 in tumor tissue.*

For the first time, breast cancer was analyzed for disease-free survival versus nuclear expression of RIPK3. No such data were found in the literature. Won et al. (2021) reported that disease-free survival in breast cancer patients is lower when tumor cells have low cytoplasmic RIPK3 expression compared to high-expression patients. The results of Feng et al. (2015) in colorectal cancer. Based on data reported by Weber et al. (2018) on the possibility of RIPK3 passing from the nucleus into the cytoplasm of cells and vice versa, we can again assume that the high nuclear expression of RIPK3 in breast cancer means that retention of necroptotic protein in the nucleus of tumor cells avoids the formation of necrosome in the cytoplasm and, accordingly, no cell death occurs. This could be considered as a possible mechanism of tumor cells for long-term survival. If we accept this hypothesis as true, our results on the relationship between nuclear expression of RIPK3 in tumor cells and disease-free survival can be considered consistent with data from studies by Won et al. (2021) and Feng et al. (2015).

V. CONCLUSION

The incidence and mortality from breast cancer continue to increase worldwide with each passing year. Although the survival of patients with this disease is higher than that of other solid tumors, the high incidence, younger age at diagnosis, aggressiveness of the tumor, early lymph node metastases and poor prognosis in patients with breast cancer, especially in those with a triple-negative variant of the tumor, they cannot be ignored. Due to the heterogeneity of mammary carcinoma and the complex mechanisms for its regulation, biomedical treatment strategies face major challenges in clinical practice. Currently known basic strategies for the treatment of the disease, such as surgery, chemotherapy, radiation therapy, immunotherapy and hormone therapy, may not lead to the complete elimination of the tumor, but are also associated with causing adverse side effects. Innovative new therapies are needed, especially for patients with ineffective treatment, such as those with triple-negative breast cancer and metastatic disease. Activation of cell death can be considered as a possible new option for the treatment of malignancies in general. Necroptosis is a type of necrotic form of programmed cell death that has potent immunogenicity and is involved in complex interactions with other types of cell death such as autophagy and apoptosis. There is growing evidence that it plays an important role in the progression and metastasis of tumors, the immune response against malignant cells and the prognosis of patients with malignant diseases. Stimulation of necroptosis by various drugs and chemicals that cause or manipulate the necroptotic pathway is emerging as a new approach to overcoming tumor cell resistance to apoptosis and maintaining antitumor immunity in the treatment of malignancies. Data on the influence of necroptosis on the development and progression of breast cancer are very scarce. There have been isolated reports that low expression of RIPK3 is associated with aggressive clinical features, while high expression of RIPK3 is associated with better survival, with the marker of necroptosis being an independent prognostic factor. Further in-depth studies are needed to investigate the role of necroptosis in the

development, metastasis, and future treatment of breast cancer patients.

VI. CONCLUSIONS

1. Breast cancer, at the time of diagnosis, is most often found between the ages of 61 and 70 years, in most cases is in the T2 N0 stage, shows a moderate degree of differentiation and high Ki67.

2. After adjuvant hormone, radiation and / or chemotherapy and remission, the median progression-free survival of breast cancer patients is 113.8 months.

3. Cytoplasmic expression of RIPK3 in mammary carcinoma tumor tissue is lower, while nuclear expression is higher than in the control group.

4. The cytoplasmic expression of RIPK3 in lobular carcinoma is higher than in ductal carcinoma.

5. Cytoplasmic RIPK3 expression is lowest in triple-negative carcinomas.

6. Cytoplasmic expression of RIPK3 in mammary carcinoma tumor tissue did not indicate dependence on patient age, T-stage, tumor spread (metastasis), tumor HER2 status, and was not associated with patient survival.

7. Highly differentiated mammary carcinomas have higher values of cytoplasmic expression of RIPK3 compared to tumors with a low degree of differentiation.

8. Intense cytoplasmic expression of RIPK3 in breast cancer occurs at pronounced intensity and high total score of ER, at expressed expression by area and intensity and high total score of PR and at low expression of Ki67.

9. Nuclear expression of RIPK3 in mammary carcinoma tumor tissue did not show dependence on patient age, histological type of carcinoma, T-stage, and degree of tumor differentiation.

10. With high nuclear expression of RIPK3 in tumor cells, the tumor is more likely to have lymph node metastases.

11. Nuclear expression of RIPK3 in mammary carcinoma tumor tissue does not show a dependence on the area, intensity and total score of ER and PR, as well as on the HER2 status of the tumor and the proliferative marker Ki67.

12. High nuclear expression of RIPK3 in tumor tissue is associated with low progression-free survival in patients with breast cancer.

VII. CONTRIBUTIONS

Scientific contributions of original character

1. A complex clinical-morphological and IHC analysis of the receptor status and RIPK3, a marker for necroptosis, was performed in patients with breast cancer.

2. An analysis of the immunohistochemical expression of the marker for necroptosis RIPK3 was performed in order to clarify its role in the prognosis and survival in patients with breast cancer.

Scientific contributions of practical and applied nature

1. For the first time in our country the prognostic and predictive value of RIPK3 in patients with breast cancer has been determined.

2. RIPK3 expression was assessed in relation to the proliferative marker Ki67 and the receptor status of breast cancer patients.

3. RIPK3 expression was analyzed in relation to clinical and morphological parameters to determine the risk of metastasis.

VIII. PUBLICATIONS

Full text articles:

1. Stoeva M. New aspects in the classification of epithelial tumors of the mammary gland. Varna Medical Forum, 2021; Vol. 10 (2): 118-123.

2. Stoeva M., Stoev L., Tzaneva M. Necroptosis-molecular mechanisms and role in tumor growth. Varna Medical Forum, 2021; Vol. 10 (2): 124-129.

Participations:

1. M. Stoeva, L. Stoev, M. Tzaneva. Expression of necroptosis marker RIPK3 in breast cancer: a clinico-morphological and immunohistochemical analysis. IMAB, 2021