

MEDICAL UNIVERSITY - PLEVEN
FACULTY OF HEALTHCARE
DEPARTMENT OF "SURGICAL NURSING CARE"

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Studying the incidence and role of morphological and biological tumor
heterogeneity in breast carcinoma

ABSTRACT

For the award of an educational and scientific degree
"doctor"

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The dissertation is developed on 145 pages, contains 2 diagrams, 16 figures, 36 tables and 4 appendices. The bibliography includes 358 sources, most of them from the last 10 years, and 87 from the last 5 years.

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Abbreviations used

NACHT- Neoadjuvant chemotherapy
pCR- pathological complete response
ER- estrogen receptor
PgR- progesterone receptor
HER2- human epidermal growth factor receptor 2
Ki 67- proliferation indeks
G- tumor grade
TNBC- triple negative breast cancer
WHO- World Health organisation
NOS- not otherwise specified
NST- no special type
AR- androgen receptor
DFS- disease free survival
OS- overall survival
CNB- core needle biopsy
MRI- Magnetic resonance imaging
BIRADS- Breast Imaging Reporting and Data System
EC- Epirubicin + Endoxan
TNM- tumor, node, metastases
cT- clinically assessed tumor
cN- clinically assessed lymph nodes
ypT- Pathoanatomical assessment of tumor size after NAHT
ypN- pathoanatomical evaluation of lymph nodes after NAHT
DCIS- ductal carcinoma in situ
ISH – in situ hybridization
FISH- Fluorescence in situ hybridization

Background:

Breast cancer is the most common oncological disease in women and ranks first in terms of mortality in Bulgaria. In recent years, considerable material has been collected proving that breast carcinoma is an extremely heterogeneous disease at clinical, morphologic, genomic level, manifesting clinically with different behavior and different response to therapy. This requires, in addition to the universal morphological subtyping, other markers to be added. Such biomarkers, available in breast cancer are - estrogen receptor (ER), progesterone receptor (PgR), human epidermal growth factor receptor 2 (HER2), as well as others, with independent prognostic significance - Ki 67 proliferation index, differentiation grade G or those with more scientific weight such as gene expression profiling. Caspase-3 has been investigated in recent years, mostly in combination with Ki 67 proliferation index, having an additional negative predictive value on overall survival. Tumor heterogeneity - morphological or based on biomarker expression - is associated with poorer prognosis and survival and is considered a major cause of therapeutic resistance. The potential of a given therapy to eradicate the tumor mass and its metastases depends on the ability to overcome the heterogeneous response of individual cell lines, which is a multifactorial process and reflects all forms of tumor heterogeneity. Inter and intratumor heterogeneity, temporal and spatial differences in phenotypic and genotypic aspect manifested in the course of tumor evolution, impose the need of developing an individualized approach for breast cancer patients to achieve optimal therapeutic results.

II. AIMS

- To establish the role of morphological and biological tumor heterogeneity in the diagnosis of breast cancer patients suitable for neoadjuvant chemotherapy
- To determine its role on the subsequent therapeutic approach

III. Propositions

1. To determine the incidence of tumor heterogeneity in breast cancer patients
2. To determine the dependence of the frequency of tumor heterogeneity on the method used for histological verification
3. To determine the impact of neoadjuvant chemotherapy on tumor heterogeneity
4. To determine recurrence and survival in patients with proven tumor heterogeneity and neoadjuvant chemotherapy
5. To determine the role of proliferative and apoptotic activity before and after neoadjuvant chemotherapy in a tumor population containing cases with tumor heterogeneity

IV. Materials and Methods

1. Materials used on the assigned tasks:

To fulfill the set goals, we conducted a retrospective study with random selection of participants according to disease and therapy. The study covers a period of 5 years /2016r.-2021r./. During screening, two cases (adult patients) with tumor heterogeneity were identified, whose characteristics make it difficult to standardize the therapeutic behavior. The latter are described and documented in detail in the present study without additional research in order to fully and objectively present the overall heterogeneity in the studied general population.

110 adult patients were studied, with clinical and imaging data (categorized with BIRADS 4-5) of formation in the mammary glands subject of histological verification, after which they meet the criteria for neoadjuvant chemotherapy (NACHT) - patients with advanced disease suitable for preoperative systemic therapy. All patients included in the study were diagnosed and surgically treated after NACHT in UMBAL-Pleven and were randomly selected from the archive of the Department of Clinical Pathology at UMBAL-Pleven.

The following were accepted as exclusion criteria

- Patients in whom the histological diagnosis or surgery after neoadjuvant chemotherapy was not performed in UMBAL - Pleven due to a difference in the patho-anatomical responses from the different units.

- Patients with depleted or missing histological materials

- Minor patients

- Deprived of liberty

Factors such as sex, age, focality, tumor heterogeneity, histological variant/s, histological grade on diagnostic biopsy and resectate after neoadjuvant chemotherapy were evaluated. The therapeutic approach is also taken into account.

The NAHT was performed according to recent guidelines.

- In HER2 positive patients – Pertuzumab + Trastuzumab + Docetaxel

- In HER2 negative patients – Epirubicin + Endoxan

- In triple negative patients - Paclitaxel + Carboplatin

The therapy of all heterogeneous tumors is directed against the more aggressive component.

The patients with tumor heterogeneity were identified - after needle biopsy or excisional biopsy before NACHT and were evaluated on resectate after NACHT.

Clinical and basic prognostic parameters, included in the pathoanatomical assessment of patients during the primary diagnosis and subsequent radical surgical treatment after neoadjuvant chemotherapy, were analyzed.

For the purposes of the study, when performing tasks 3 and 4, patients with established tumor heterogeneity - after needle biopsy or resectate were accepted as the main group. A control group was formed, composed of randomly selected patients without heterogeneity from the initial study group matched for age, gender and morphological variant of one of the tumor components (NST or lobular carcinoma), as close as possible to the more aggressive component.

In the results obtained by us, the impact of standard variables of TNM classification of tumors, Grade and molecular markers in both groups / with and without available heterogeneity / is tracked. Size and infiltration of the primary tumor / cT / was determined based on imaging and clinical data. Due to the unclear borders of some tumors, it has a relatively indicative character. For the purposes of the study, patients were divided into two groups:

- tumors up to 2 cm in size and
- tumors larger than 2 cm, including tumors affecting the skin and chest wall, as well as orange peel skin.

For multifocal tumors, staging is based on the largest tumor mass. Bilateral carcinomas are staged separately, with the larger of the two tumor sizes accepted for analysis.

Selected patient groups were matched statistically.

In task 5, levels of Ki-67 and caspase-3 expressed on diagnostic biopsy were correlated with degree of differentiation, surrogate molecular subtype, T, N variables, as well as ypT, ypN, assessed by Sataloff on resectate.

The data presented are the result of an accomplished project financed by the Pleven University of Medicine - Project No. 6/2022.

Permission for the research was obtained from the Ethics Committee of the Medical University of Pleven with number 707-KENID/ 03.06.2022. (Application № 3)

2. Methods used on the assigned tasks

2.1 Surgical

2.1.1 Core needle biopsy /CNB/- Performed under ultrasound control with a 14-gauge semi-automatic and automatic punch needle. The cylinders taken were between 3 and 6 from different parts of the lesion. Each core is numbered according to order of collection and fixed in formalin according to unified recommendations.

2.1.2 Excisional biopsy - diagnostic excision, in case of impossibility to conduct CNB, in diagnosis of the disease before NACHT, meeting the latest standards for diagnosis in patients with breast cancer.

2.1.3 Radical surgical treatment - Breast-conserving surgery or Modified radical mastectomy /MRM/ after NACHT according to the accepted indications/contraindications for organ-preserving surgeries in patients with breast cancer.

2.2 Pathoanatomical methods

Basic protocols for working with tissue samples were followed - for fine-needle biopsies and resectates, respectively

Morphological subtyping, staging and grade, as well as determination of mixed (morphologically heterogeneous) tumors were performed according to WHO on sections stained according to a routine protocol with Hematoxylin Eosin and, if necessary, immunohistochemical verification or staining with another histochemical method (PAS).

In determining the receptor status of the tumors, antibodies from respective clones ER (clone EP1) Dako, PgR (PgR 636) Dako, E-cadherin (NCH-38) Dako, EMA clone E29, Ki-67 (MIB-1, Dako), Ki-67 (Ki-67 rabbit antibody monoclonal clone SP6/Biocrine USA), HER2 (4B5) Dako, caspase-3 /rabbit polyclonal antibody/Biocrine USA/; using envision visualization or HercepTest™, Dako (kit). Staining was performed on an autostainer Dako link 48.

In situ hybridization (ISH) for HER2 was performed in external laboratories. The result is entered in the clinical documentation, and is interpreted binary (positive/negative status) based on clinical recommendations.

Reporting of results for steroid receptors and HER2 was performed according to the established recommendations for the period.

Ki-67 expression was scored according to current recommendations with counting of a minimum of 500 cells for each of the components scored as positive when staining was present, regardless of intensity.

Caspase-3 expression was assessed according to recommendations by Engels et al, 2013 with levels > 0.5% considered elevated.

Surrogate molecular subtypes are defined based on recommendations published by WHO.

2.3 Documentary method was used in task 4:

Cancer progression and recurrence were assessed using a hospital electronic system database. Information on overall survival was obtained from the national registry database.

2.4 Statistical methods - a number of parametric and non-parametric tests were used, according to the analyzed variables.

2.4.1 When working on task 1: The comparison by age was performed using the Signed rank test, with $P < 0.05$ being considered significant. Statistical methods (ANOVA) were used to assess a statistically significant difference in age between patients with and without tumor heterogeneity, after assessment of normality of distribution. The relationship between non-parametric variables (focality and heterogeneity) was examined using a χ^2 test. Values of $p < 0.05$ were considered statistically significant. Statistical analysis used SPSS software.

2.4.2 When working on task 2: Sensitivity and specificity of core biopsy to detect tumor heterogeneity in breast carcinoma were determined according to the approach reviewed by Monaghan TF et al, 2021 by calculating Cohen's Kappa. Heterogeneous patient grouping based on diagnostic approach was analyzed according to the non-parametric Kruskal-Wallis method due to lack of normal distribution. Values of $p < 0.05$ were considered statistically significant. Data were processed and analyzed by SPSS computer software.

2.4.3 When working on task 3: The comparison of grade in the group of heterogeneous tumors and non-heterogeneous tumors, respectively, before and after neoadjuvant therapy was carried out by means of the Marginal Homogeneity test / Stuart-Maxwell χ^2 test, with $P < 0.05$ being considered significant, the calculations were carried out using software. /John Uebersax PhDemail: jsuebersax@yahoo.com, web: <http://ourworld.compuserve.com/homepages/jsuebersax/>.

Comparing grade of malignancy before neoadjuvant chemotherapy, presence of molecular subtype change in the context of neoadjuvant chemotherapy, comparing tumor size and nodal status after neoadjuvant chemotherapy, as well as the therapeutic effect on tumor and nodal status (reported by Sataloff), between the group of heterogeneous and non-heterogeneous tumors was performed using The Fisher's Exact test, and the χ^2 test with $P < 0.05$ considered statistically significant (calculated with the SPSS program).

Determining the therapeutic effect was performed according to Sataloff for patients from both groups, and in the context of changing trends, the tumor effect was also counted binary - as complete/almost complete pathological response (with residual single tumor cells and groups of cells) and incomplete therapeutic response for all other cases.

2.4.4 When working on task 4: Survival parameters were analyzed using the Kaplan-Meier method and summarized with median and 95% confidence intervals. Survival curves were compared using log-rank tests. The survival period was measured from the time of diagnosis until the end of the reference period when the data were collected. A primary endpoint was to determine disease-free survival and overall survival limited by all-cause death. Statistical software was used./ <https://www.statskingdom.com/kaplan-meier.html>; <https://www.bmj.com/about-bmj/resources-readers/publications/statistics-square-one/12-survival-analysis/>. The patients who dropped out are censored.

2.4.5 When working on task 5: ANOVA F analysis of variance or one-way non-parametric Kruskal-Wallis test with a confidence interval of 95% was used to establish the dependence of Ki-67 on the studied variables. Values of $p < 0.05$ were considered statistically significant. When establishing the dependence of Caspase 3 on the studied variables, the χ^2 test was also used with values of $p < 0.05$ considered statistically significant, backed up by The Fisher's Exact test.

The statistical information from the investigated markers in both cases was processed and analyzed by SPSS computer software.

V. Results and discussion:

1. Task 1: To determine the incidence of tumor heterogeneity in breast cancer

1.1 General characteristics of the examined patients by age and sex, with determination of the frequency of tumor heterogeneity and multifocality

Of the included patients - 108 were women (98.18%) and two were men (1.82%). –

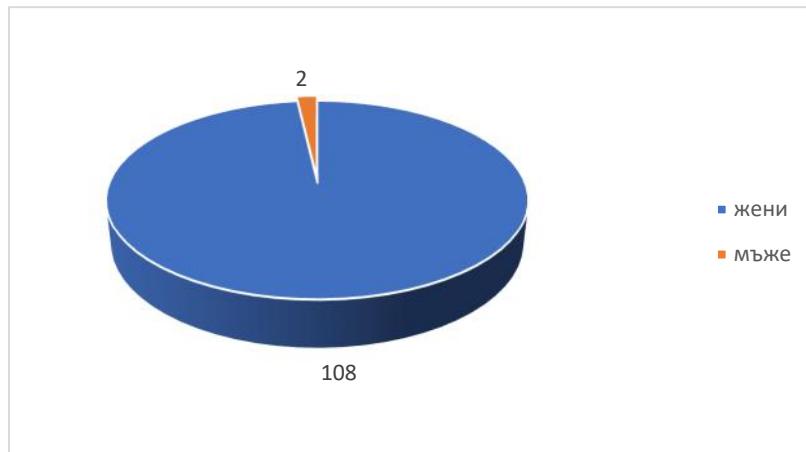


Diagram 1 graphical presentation of gender ratio

The age in the presented results ranged between 30 and 82 years with a mean age of 59.22 years, largely consistent with the data published to date in the literature. Data state that breast cancer in over 80% of cases are in the age group ≥ 50 years with a mean age of 62 years. / summarised by Giaquinto AN et al, 2022 /. Of the examined 110 patients, with proven heterogeneity / presence of two or more demarcated tumor foci in one or both breasts / on diagnostic biopsy and resectate were 12 cases or 10.91%.

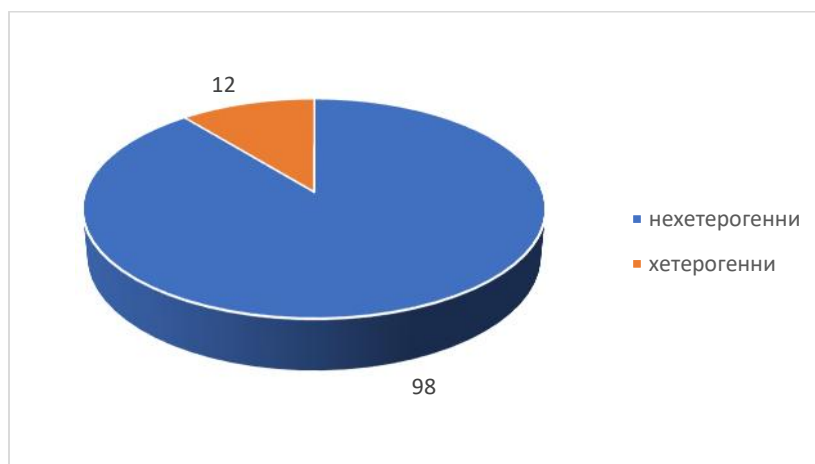


Diagram 2 graphical presentation of the distribution by number of heterogeneous to non-heterogeneous

The frequency obtained by us is included in the interval between 9% and 15% described in the literature for cases in which the type of tumors cannot be referred to the standard diagnostic groups. Variations in tumor morphologic and/or molecular expression underlie intratumoral heterogeneity and cause diagnostic difficulties.

In terms of age distribution, patients with tumor heterogeneity had a mean age of 50.58 years (SD +/- 17.1383) and in the group without heterogeneity the mean age was 60.26 years (SD +/- 12.5938).

When comparing age of patients with tumor heterogeneity (found on CNB and resectate) and those with non-heterogeneous tumors was found that patients with heterogeneous tumors were significantly younger $F=5.8020$, $p=0.0177$ (Table 1)

Table 1 Comparison of patients with and without tumor heterogeneity by age

	n	Median age	SD	min /max
patients without tumor heterogeneity	98	60.2551	12.5938	30-82
patients with tumor heterogeneity	12	50.5833	17.1383	31-73

Our results support the data described in the literature on the peculiarities of breast cancer at young age. This group is characterized by higher degree of heterogeneity, the frequency of which increases with years and reaches the highest levels in the range of 40-49 years:

1.2 Characteristics of the heterogeneous group of patients

According to our results, breast cancer patients affected by tumor heterogeneity were only women. It cannot be excluded that this is due to the small number of men included in the sample, but tumor heterogeneity was not observed in men.

Two of the cases with heterogeneity involved synchronous carcinoma affecting both breasts. In the remaining ten cases, it concerns tumor foci in one breast, and in three of the cases the tumor is multifocal and forms two tumor foci.

In ten of the cases with heterogeneity, it concerns morphological with or without additionally recognizable molecular heterogeneity on histological examination on preparations stained with hematoxylin and eosin.

The most common combination involves two morphologically distinct components

- NST combined with mucinous in three of the cases, and in only one we have differences in the molecular expression - a combination of luminal A in the mucinous and luminal B (HER 2 variant) for the NST component.

Figure 1 shows HER 2 luminal B type in mixed mucinous and NST (ductal) carcinoma G2 on CNB before NACHT.

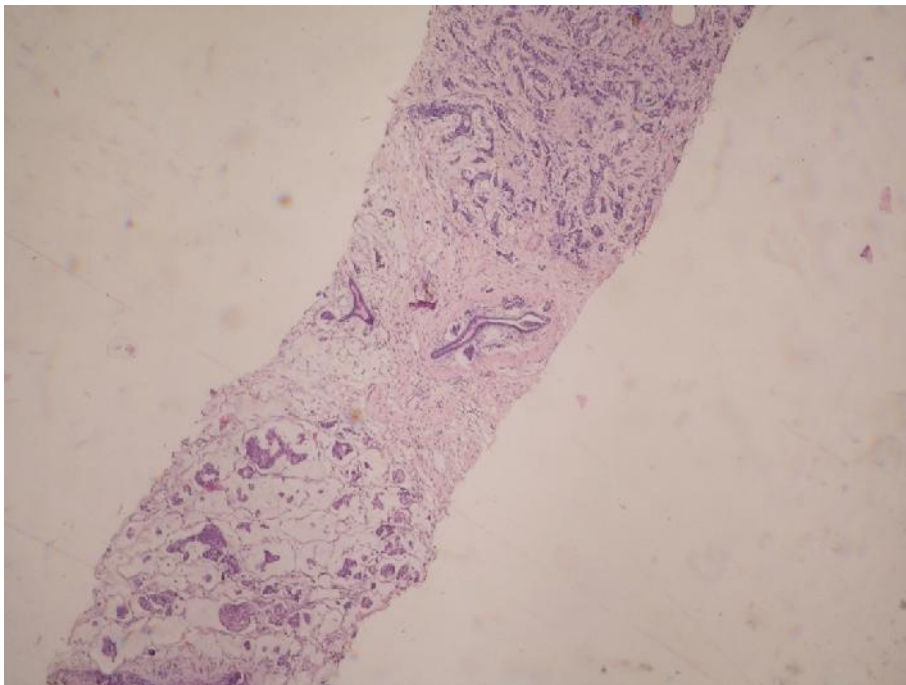


Fig. 1 Material from a fine needle biopsy represented by two components - mucinous and NST (ductal) carcinoma G2 (HE 10x)

Figure 2 shows the two-component tumor - NST G2 and mucinous carcinoma hypercellular variant after neoadjuvant chemotherapy

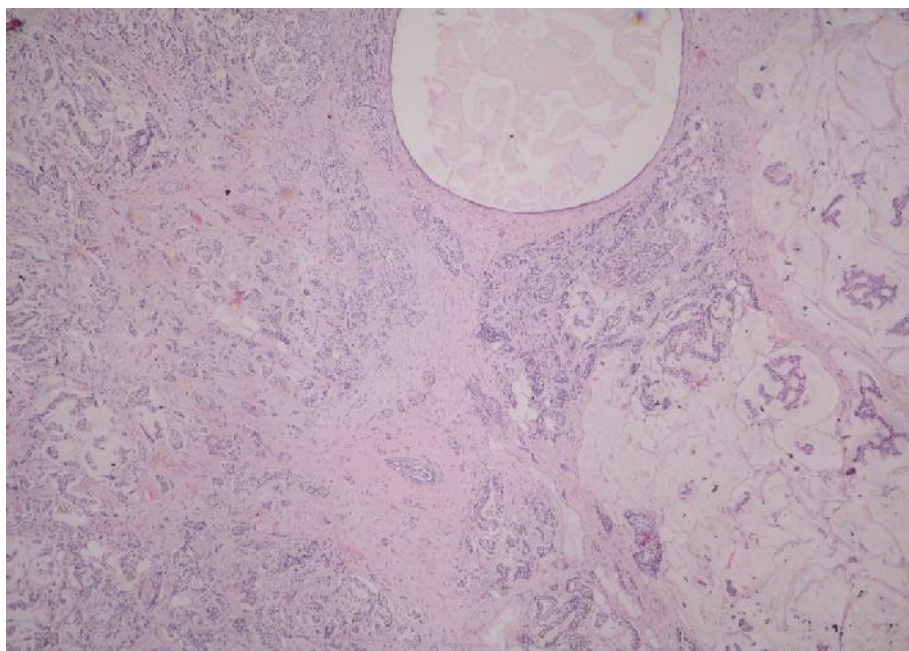
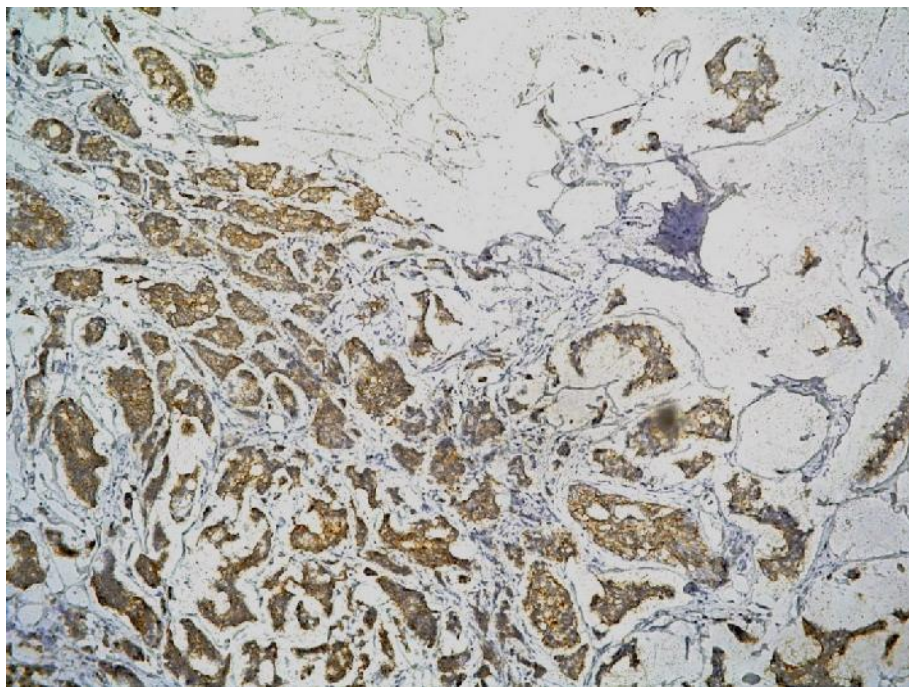
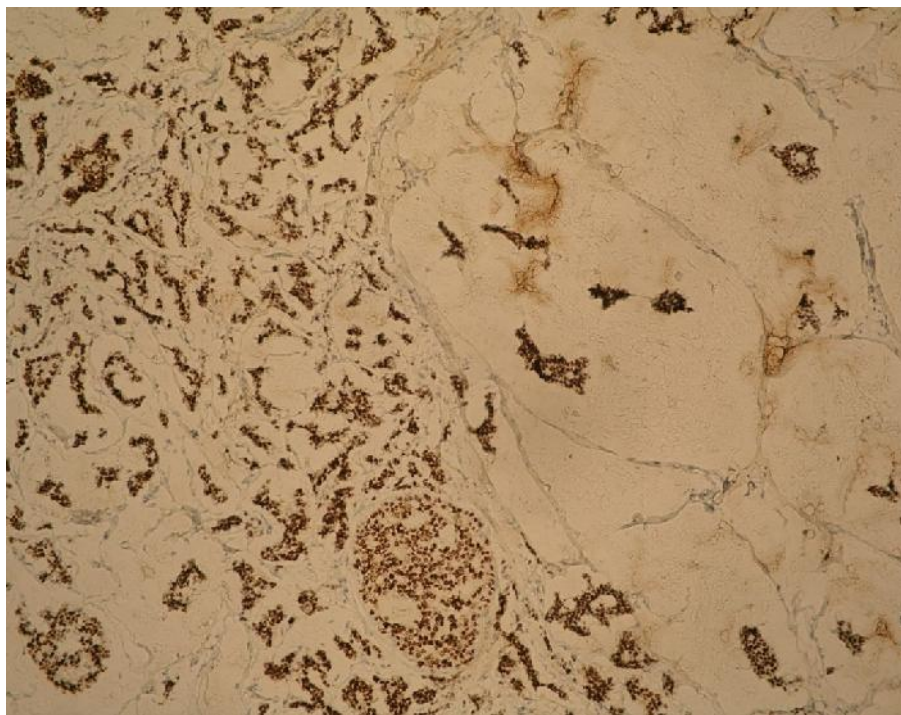


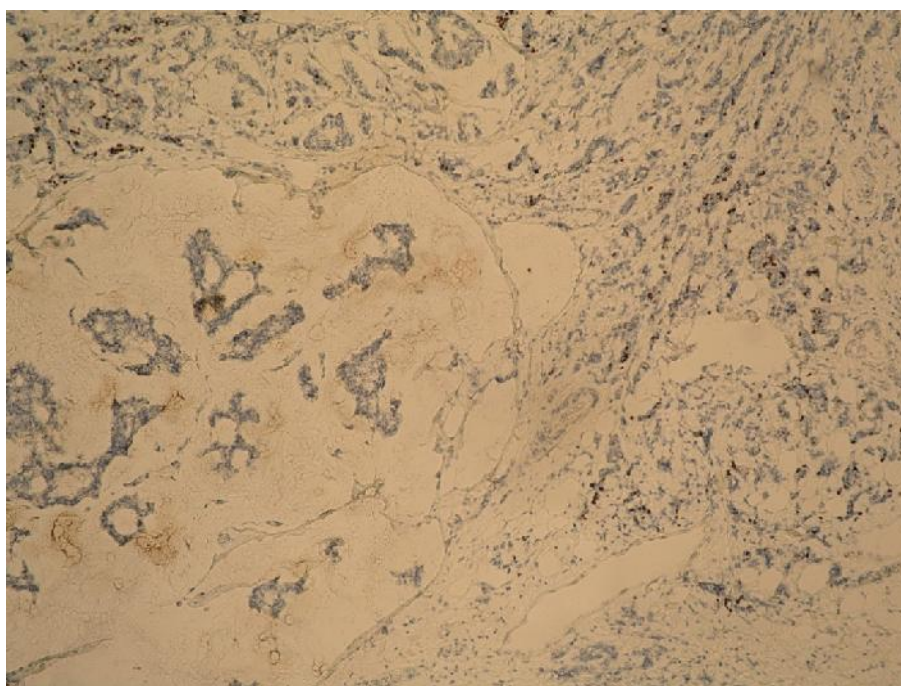
Fig 2.1 material from a resectate - both tumor components persist with a predominance of the mucinous on the background of unexpressed fibrosis and cellular stroma and the presence of a cytopathic effect. (HE 10x)



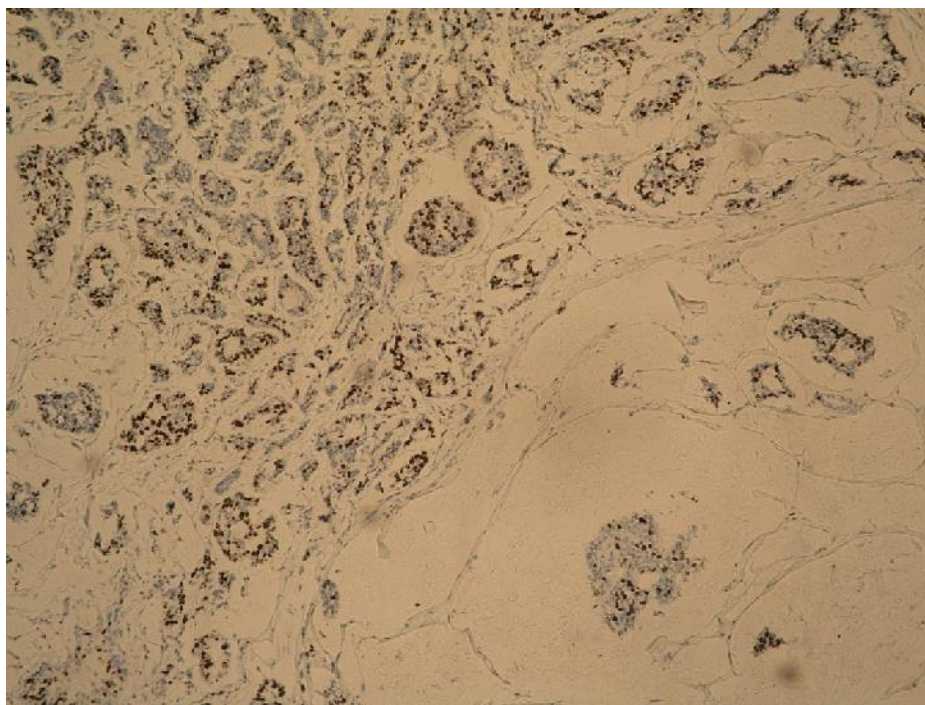
Фиг 2.2 HER2 -3+ Strong complete membranous staining in more than 10% of tumor cells (HER 2, 40x)



Фиг 2.3 ER Strong complete membranous staining in more than 10% of tumor cells (ER 100x)



Фиг 2.4 receptor - moderate nuclear positivity in about 15% of tumor cells (PgR100x)



Фиг 2.5 Ki 67- positivity in over 30% of tumor cells (Ki 67, 40x)

Mucinous carcinomas are rarely HER 2 positive. As in the case described, HER 2 expression is carried by the NST component. The morphological variant does not determine a different path of stimulation of tumor growth, respectively it is not decisive for the therapeutic choice. In the specific case, it is influenced by the receptor status of the tumor as a whole and not by its different morphological components.

- Invasive ductal NST combined with lobular in three of the cases, and only in one case we have differences in the molecular expression - triple negative for the NST and luminal A for the lobular component.

- NST carcinoma in combination with metaplastic carcinoma in two of the cases and in both cases differences were also observed in the molecular expression - luminal B type expression for the NST region and triple negative for the metaplastic component

- NST carcinoma in combination with micropapillary carcinoma in one case, with the micropapillary being luminal B (HER 2 variant) and the residual NST being luminal B type

Figure 3 shows a core biopsy showing the micropapillary and ductal NST G2 component.

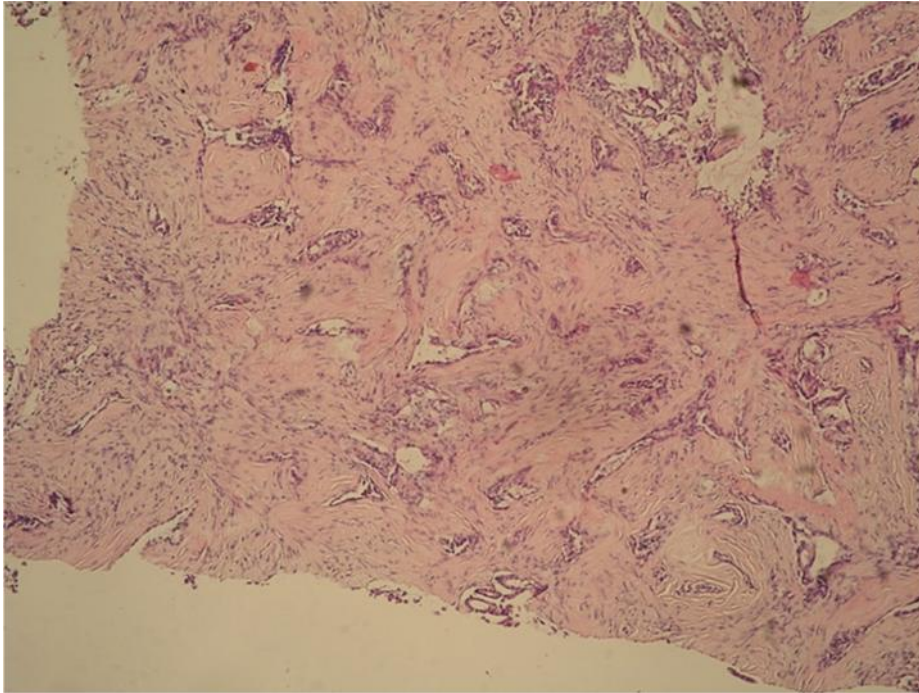


Fig 3.1 Fine needle biopsy material showing the micropapillary and nested component determined as ductal NST G2 and DCIS – G2 carcinoma (HE 10x, 400x)

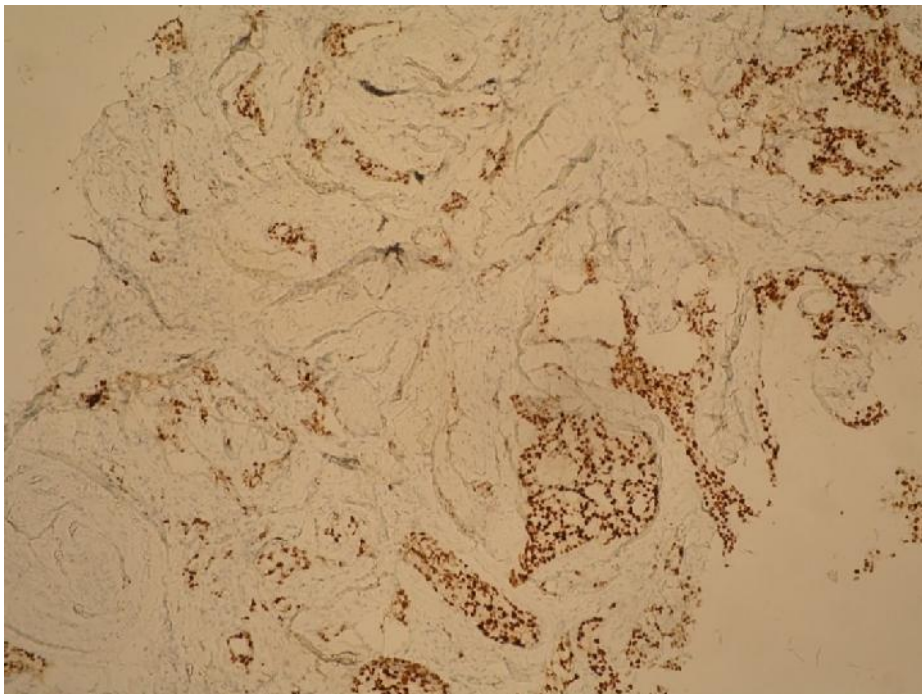


Fig 3.2 ER-receptor - nuclear positivity in about 80% of tumor cells (ER 100x)

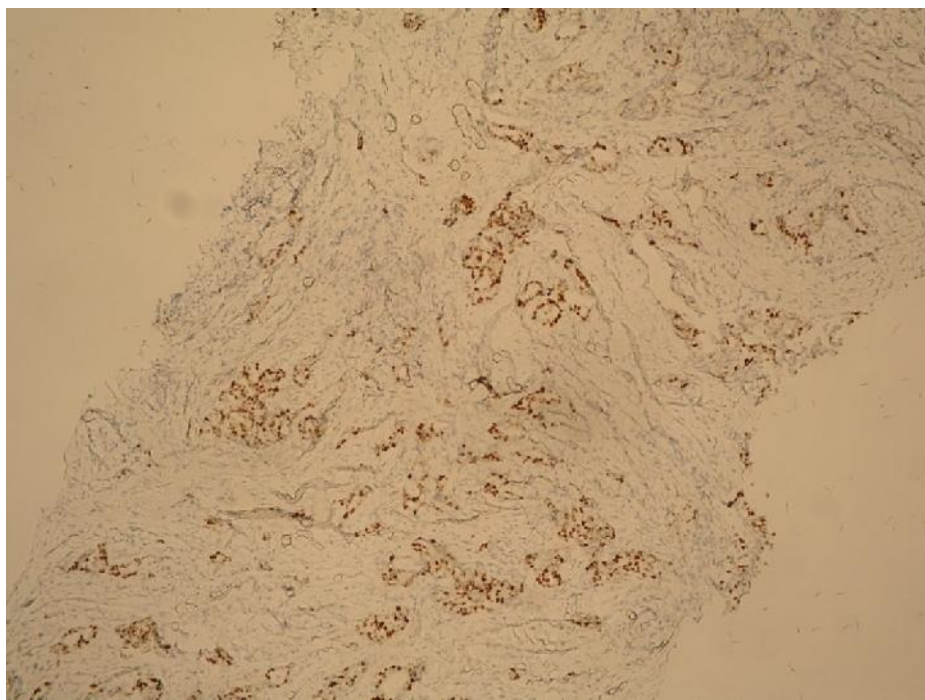
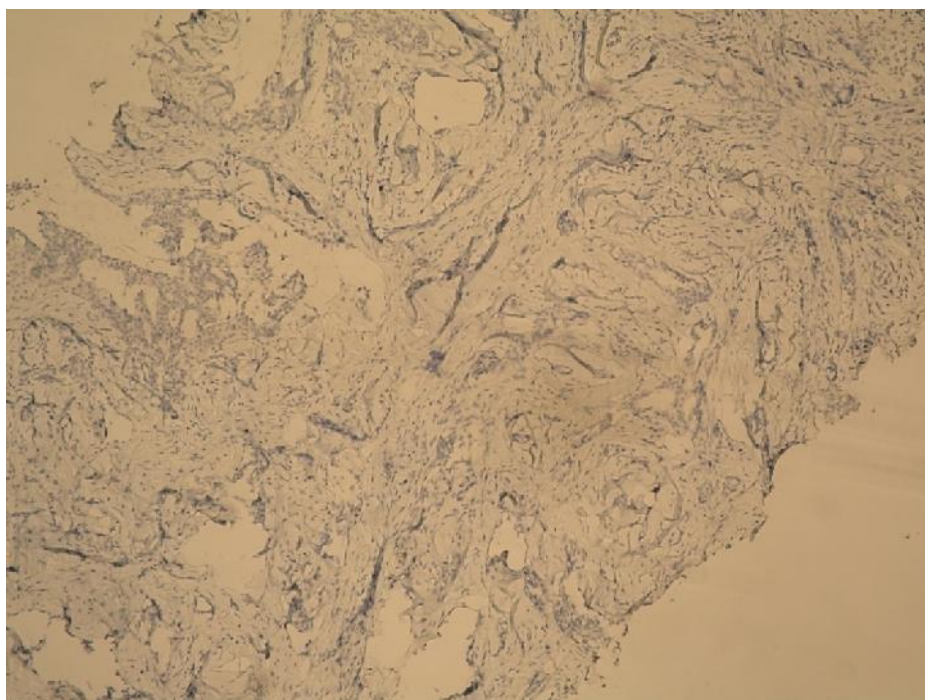
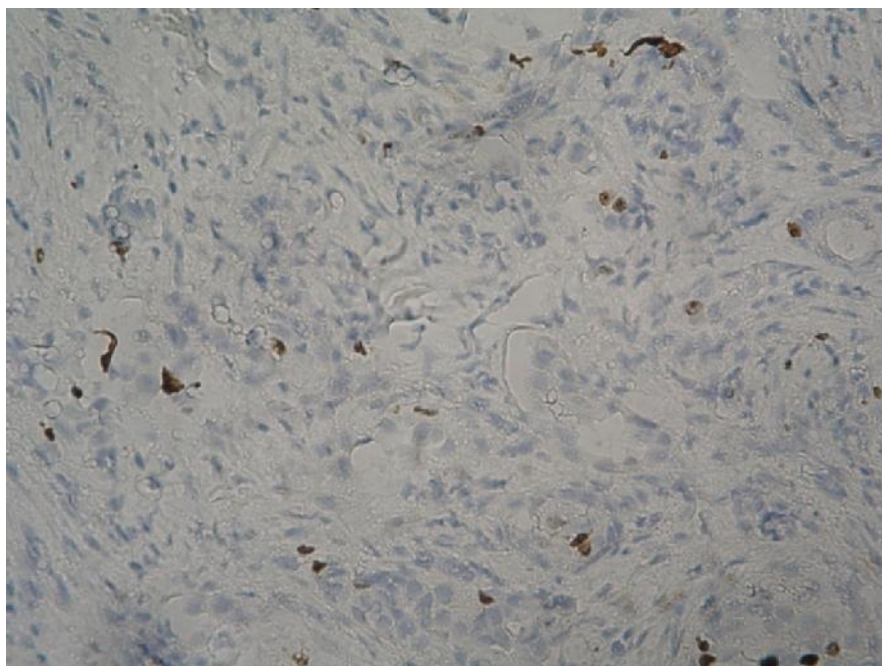


Fig 3.3 PgR receptor moderate nuclear positivity in about 60% of tumor cells (PgR, 100x)



Фиг 3.4 HER 2 – negative (HER2, 100x)



Фиг 3.5 Ki 67 positivity in about 10% of tumor cells (Ki 67, 100x)

The case is highly debatable due to the presence of a marked micropapillary component, but no distinct morphologic criteria for the diagnosis of micropapillary carcinoma. It is clearly demonstrated how the small volume of tissue can distort the image of the overall appearance of the tumor. The present case was considered to be a heterogeneous tumor after the complete examination of the material. Tumor components remain within the luminal type. Micropapillary carcinoma is perceived as a more aggressive tumor, with a worse prognosis, and its presence in a sufficient volume of the resection is associated with the presence of a higher proliferative activity and accordingly a luminal B type of the tumor.

In our study, the frequency of tumors with two components in the form of lobular and ductal carcinoma, which is also the most frequently conjugated combination in the literature, is almost twice lower than that described in scientific publications, on account of other less common variants.

A combination of three morphologically distinguishable components was observed in one case

- NST ductal carcinoma, mucinous and micropapillary carcinoma that contain distinct luminal A components in the mucinous component and luminal B type in the NST and micropapillary component.

Figure 4 presents NST G2, mucinous and micropapillary carcinoma before and after NACHT

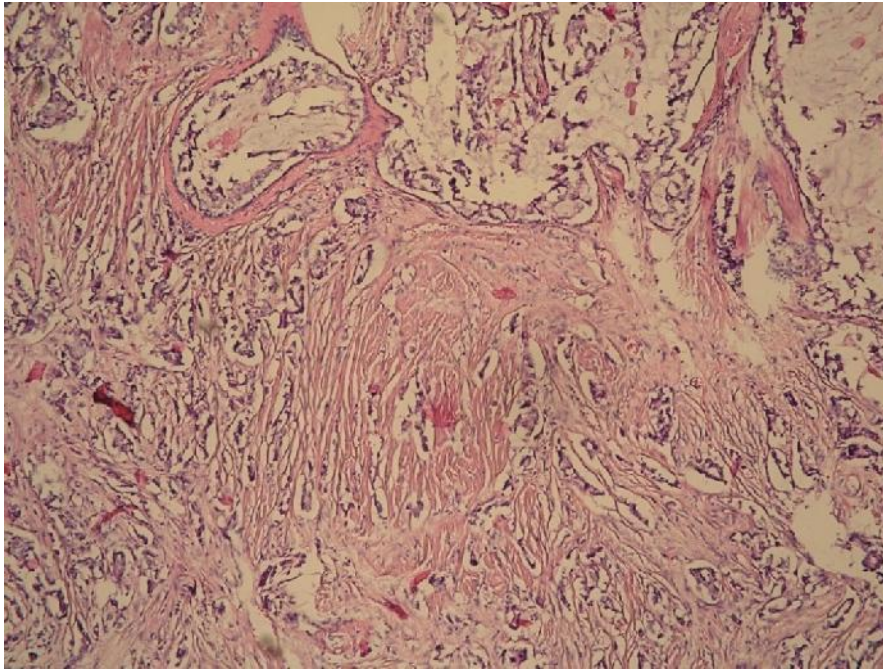
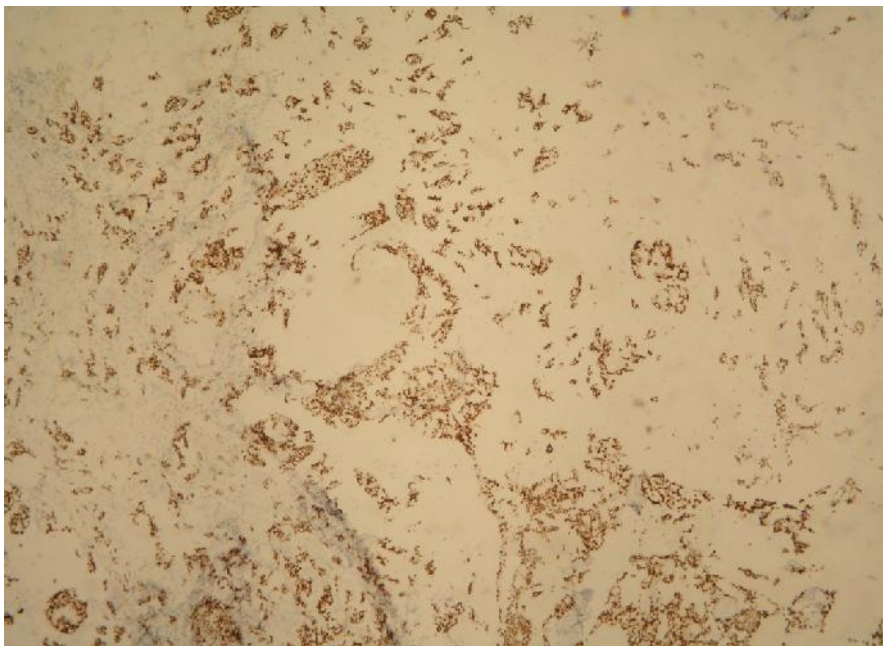
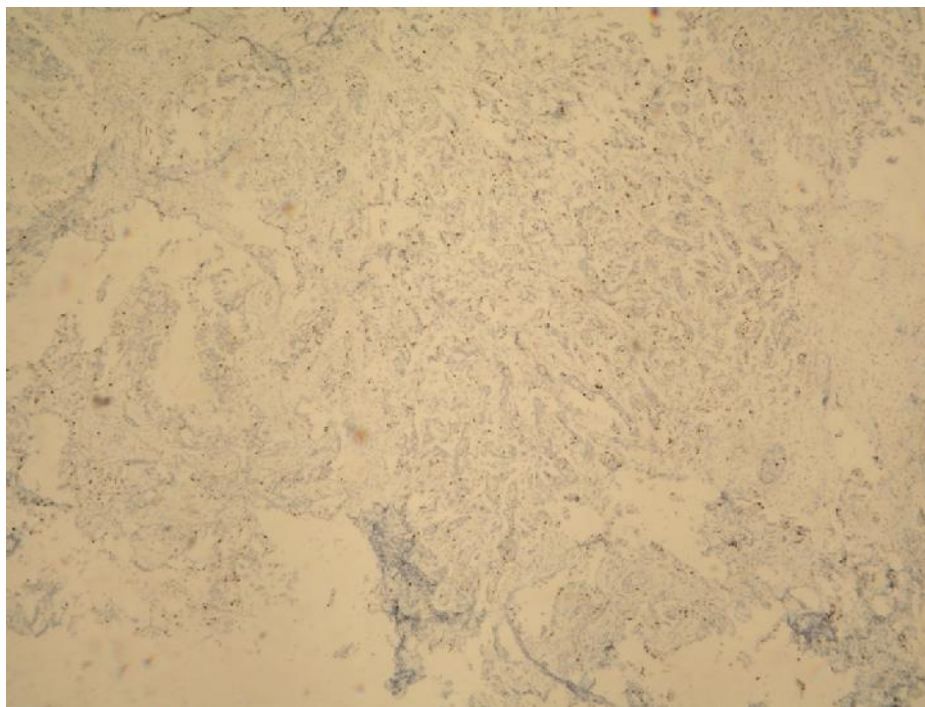


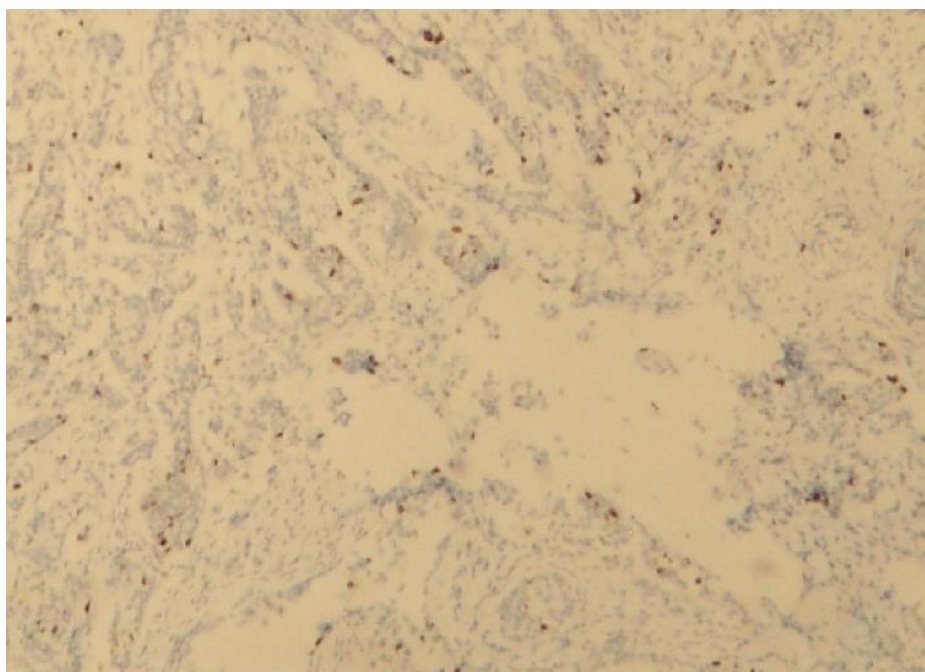
Fig 4.1 Persistence of three tumor components against the background of mild fibrosis and cellular stroma with the presence of a cytopathic effect. (HE 100x)



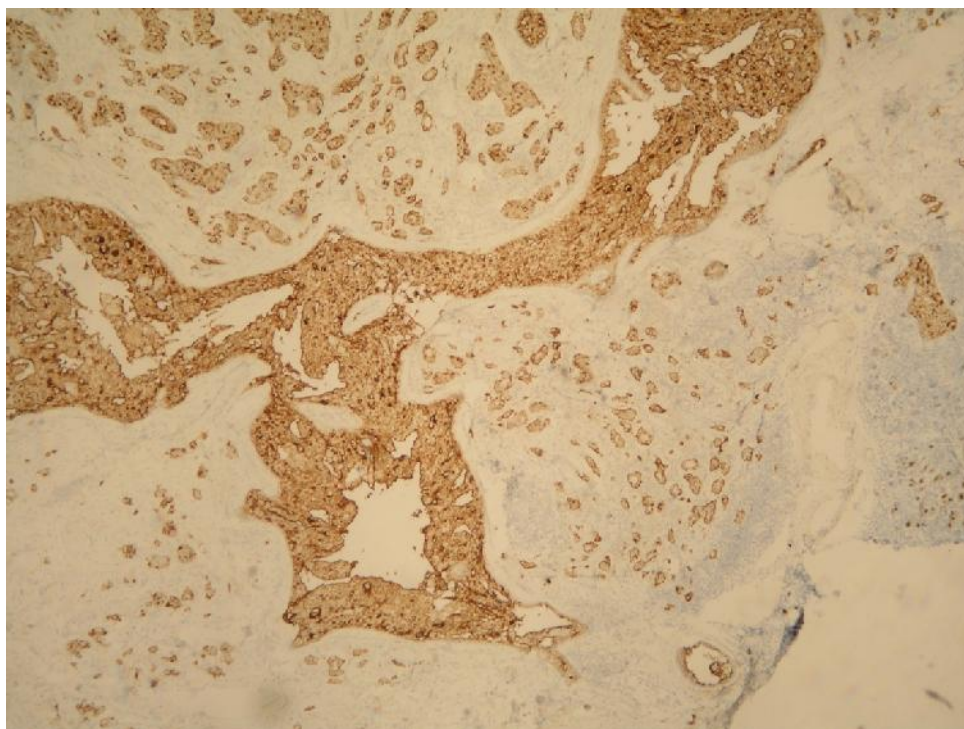
Фиг 4.2 ER receptor - nuclear positivity in about 80% of tumor cells (ER 100x)



Фиг 4.3 PgR receptor - moderate nuclear positivity in about 10% of tumor cells (PgR100x)



Фиг 4.4 Ki 67- positivity in about 15% of tumor cells (Ki 67, 40x)



Фиг. 4.5 EMA border micropapillary structures outside (EMA, 100x)

Molecular heterogeneity was detected in only two cases after immunohistochemical examination.

- NST carcinoma with two clearly demarcated immunoprofiles luminal A type and areas of luminal B type.
- A distinct ER positive component is encountered within metaplastic carcinoma.

Table 2 shows the distribution of cases with heterogeneity according to morphological variant, focality, degree of differentiation and molecular subtype before and after NACHT.

Table 2 Distribution of cases with heterogeneity

No	age	Morphological variant based on evaluation of the tumor (CNB, Resectate and metastases)	Tumor focality	G before NACT	G after NACT	Molecular subtype before neoadjuvant therapy	Molecular subtype after neoadjuvant therapy
1	68	Morphological (ductal/lobular) Ductal - CNB; ductal /lobular- resectate	unifocal (bilateral)	3	3/2	2	2/2
2	68	Molecular and Morphological (NST with distinct variation in morphology)	multifocal	2/3	2/3	1/1	1/2
3	37	Morphological (NST and mucinous) NST - biopsy; NST and mucinous- resectate	multifocal	2	3/ -	2(HER2)	2(HER2)/1
4	73	Morphological and molecular (NST and micropapillary) micropapillary CNB; NST- resectate	unifocal	2	2/-	2(HER2)	2/2(HER2)
5	67	Morphological (NST and metaplastic) NST and metaplastic – CNB; NST - resectate	unifocal	2	3	2(HER2)	2
6	72	Morphological (NST and lobular) NST - CNB lobular - resectate	unifocal	3	2	4	1
7	41	Morphological (NST and mucinous) Simultaneous biopsy and resectate	unifocal	2/-	2/-	2(HER2)	2(HER2)*
8	37	Morphological (NST with distinct variation in morphology)	multifocal	2	2	2(HER2)	1
9	40	Morphological (NST and lobular) NST – biopsy; Lobular - biopsy from the other breast	multifocal - affecting both breasts	1/ lobular is not established	1/2	1	1/1
10	32	Morphological (NST and metaplastic) NST- biopsy; metaplastic - resectate	unifocal	3	3	2	4
11	31	Morphological (mucinous and NST) (two tumor components in a lymph node) mucinous - biopsy Mucinous and NST - resectate, NST only lymph node metastasis	unifocal	-/lacking NST, which is G2	-/2	1	1/1
12	41	Morphological NST, mucinous and micropapillary) NST – biopsy; NST, mucinous and micropapillary- resectate	unifocal	1/-/2	2	1	2

Legend: G grade: 1- highly differentiated, 2- moderately differentiated, 3 -low differentiated;
Molecular subtype: 1- luminal A, 2- luminal B, 2(HER2)- luminal HER2,
3- HER2 enriched, 4- TNCG *persistent mucinous component carrying HER 2 1+

The dependence of multifocality and heterogeneity is distinct:

$\chi^2=13.1108$; $df= 1$, $p<0,0001$ (Table 3).

In breast cancer without manifestations of heterogeneity, multifocality or multicentricity is demonstrated in only 10% of cases, while in the heterogeneous population this frequency is approximately 50% of cases.

Table 3 Dependence of multifocality on heterogeneity

	without heterogeneity	heterogeneity
unifocal	91 (82,73%)	7 (6,36%)
multifocal	7 (6,36%)	5 (4.55 %)

During the selection period, two cases of tumor heterogeneity were diagnosed and described, representing case studies in clinical practice where NACHT was not applicable for various reasons. The first case describes a 64-year-old woman with an asymptomatic, non-palpable lesion, assessed on screening mammography as BIRADS 4c, with clustered microcalcifications in the upper lateral quadrant of the left mammary gland. Chest X-ray and ultrasound showed no abnormalities. The resection was performed by marking with a metal thread, under mammographic control. Histological examination reports multifocal invasive carcinoma NST (ductal) G2 in the setting of extensive ductal carcinoma in situ (DCIS) G2 pT1aNxMx (ER-0, PR-0, HER 2-2+, Ki 67-40%). Following the decision of an oncology committee, radical surgical treatment was performed with subsequent histological verification from a resectate of single terminal tubules with lobular carcinoma in situ (LCIS). Accounting for tumor heterogeneity in this case – invasive ductal and lobular carcinoma in situ detection was possible after complete resectate examination.

The second case describes a 49-year-old woman with a palpable right breast mass. Assessed on mammography as BIRADS 4- a strongly lobulated shadow in the lower outer quadrant of the right breast measuring 43/27 mm. The performed diagnostic fine-needle biopsy under ultrasound control was uninformative - fibrous tissue, single normal ductal cells, erythrocytes and leukocytes were reported. The histological examination from the performed excisional biopsy presented tumor cells organized in groups, revealing a marked lymphocytic stromal reaction. Of these T-

lymphocytes are about 30% of the cell composition. ER, PgR and HER2 receptor are negative, Ki-67 is positive in about 50% of tumor cells. E-cadherin with moderately intense staining of cell membranes in 70% of tumor cells and p63 positive in approx 20% . CK 7 strongly positive in about 40% of tumor cells, CK 5/6 weakly marked in about 2% , vimentin strongly positive. CD 117 weak staining of cytoplasm in 5% of tumor cells, CD20 and CD3 positive respectively in B and T lymphocyte cell population. Pd-L1 is not studied. The morphological and immunohistochemical data described are consistent with a high-grade, poorly differentiated carcinoma with marked lymphocytic infiltration such as lymphoepithelioma-like carcinoma graded as pT2 cN0 cM0 G3. Following the decision of an oncology committee, radical surgical treatment was performed. Despite the absence of residual mass in the tumor bed, a new lesion was reported, in another quadrant of the breast, recognized as ductal carcinoma in situ DCIS G2, as well as a focus of invasive ductal carcinoma (NOS) G3, with marked lymphocytic infiltration. Immunohistochemical examination of invasive ductal carcinoma showed weak ER and PgR positive in about 10% of tumor cells, HER2 negative, Ki-67 - 30% , E-cadherin demonstrated moderately intense staining of cell membranes of tumor cells. The examined 17 lymph nodes have marked sinus histiocytosis. Tumor heterogeneity in this case was also registered only on the resectate and required a different therapeutic approach.

The cases demonstrate the significant problems and challenges that tumor heterogeneity can lead to. It is clear that it can be at the level of early and precursor lesions as well as represent extremely rare combinations of tumor variants of BC for which there are no proven diagnostic and therapeutic approaches established in practice. These data somewhat call into question the self-interpretation of the sick lobe theory and its application in organ-preserving surgery. The lower variability of nearby tumor foci has been confirmed in the scientific literature, but the high frequency of multifocality in the presence of tumor heterogeneity in our sample contradicts the idea of a common embryonic origin and linear evolution of the tumor.

2. Task 2: To determine the dependence of the frequency of tumor heterogeneity on the method used for histological verification

2.1 Dependence of the frequency of tumor heterogeneity on the number of harvested tissue cylinders:

From the obtained results, no statistical relationship was observed between the median number of tissue cylinders obtained and the detected heterogeneity on CNB.

K-W= 0.1208, P= 0.7281 (Table 4)

Table 4 Relationship between median number of tissue cylinders and heterogeneity detected on CNB

	n	median number of tissue cylinders	average number of tissue cylinders	min /max
patients without tumor heterogeneity on CNB	107	4.0000	4.3301	1-15
patients with tumor heterogeneity on CNB	3	4.0000	3.8571	1-7

There was no statistically significant relationship between the median number of tissue samples and overall heterogeneity, despite a trend to obtain 1 more tissue cylinder in the heterogeneous tumor group K-W= 0,3930; p= 0,5307 (Табл. 5)

Table 5 Relationship between median number of tissue cylinders and total heterogeneity

	n	median number of tissue cylinders	average number of tissue cylinders	min /max
patients without tumor heterogeneity	98	4.0000	4.2653	1-15
patients with tumor heterogeneity	12	5.0000	4.7500	1-12

Although it is generally accepted that a larger amount of harvested material guarantees a higher diagnostic value of the method, our results confirm the data published in the literature that securing more than 4 cores (tissue cylinders) does not give better results in diagnosis of breast cancer.

2.2 Sensitivity and specificity of individual diagnostic methods

Sensitivity of CNB for detecting tumor heterogeneity versus heterogeneity based on CNB + resectate after neoadjuvant chemotherapy is only 25%.

Specificity of CNB for detection of tumor heterogeneity versus heterogeneity based on CNB + resectate after neoadjuvant chemotherapy is 100%

Concordance between heterogeneity found on needle biopsy and total heterogeneity based on CNB + resectate in our analyzes remains low. Kappa = 0.3726, p< 0,0001 (Table 6).

Table 6 Comparison between core needle biopsy heterogeneity and established general index resectate + CNB

	without heterogeneity (general index resectate+CNB)	with heterogeneity (general index resectate+CNB)
without heterogeneity on CNB	98 (89.09 %)	9 (8,18 %)
with heterogeneity on CNB	0 (0.00%)	3 (2,73 %)

The sensitivity of resectate after NACHT to detect tumor heterogeneity versus total heterogeneity based on CNB + resectate after neoadjuvant chemotherapy reaches 91,67%

Specificity of resectate after NACHT for detection of tumor heterogeneity versus total heterogeneity based on CNB + resectate after neoadjuvant chemotherapy reaches 100%

The concordance between resectate-based heterogeneity and overall CNB+ resectate heterogeneity after neoadjuvant chemotherapy in our analyzes was high. Kappa = 0.9515, $p < 0,0001$ (Table 7)

Table 7 Comparison between resectate heterogeneity and established general index resectate + CNB

	without heterogeneity (general index resectate+CNB)	with heterogeneity (general index resectate+CNB)
without heterogeneity on resectate	98 (89,09%)	1 (0,91%)
with heterogeneity on resectate	0 (0.00%)	11 (10 %)

The concordance between the heterogeneity found on needle biopsy and that found on resectate after neoadjuvant chemotherapy in our analyses was low. The results obtained, show poor consistency when comparing the two surgical methods. Kappa = 0.254, $p < 0,0001$ (Табл. 8)

Table 8 Comparison between heterogeneity found on needle biopsy and on resectate

	without heterogeneity on resectate	heterogeneity on resectate
without heterogeneity on CNB	98 (89,09%)	9 (8,18%)
with heterogeneity on CNB	1 (0,91%)	2 (1.82 %)

To date, the literature has described a high, albeit variable, sensitivity and specificity of CNB versus surgical excision with values between 85 and 100%, and the same applies to assessment of biomarker expression between 79%-100%. /Meattini I et al, 2017/ Our data matches with the specificity values of the CNB method versus surgical excision, bearing in mind that the data in the scientific literature do not relate to heterogeneous breast tumors. This is where we consider, the differences with the sensitivity between the two methods come from - only 25%. In our case, the problem with the small volume of examined material in heterogeneous tumors always remains. The standard of 4 to 6 examined cylinders is optimal only if a complete coverage of the tumor mass is achieved. Even then, a large proportion of multifocal and synchronous lesions, which often have a different profile, remain unappreciated.

In confirmation of the temporal heterogeneity and low concordance described so far is the case we found of a heterogeneous tumor reported after core needle biopsy, which was not "confirmed" on resectate material due to a complete pathological response after neoadjuvant chemotherapy.

3. Task 3: To assess the impact of tumors with heterogeneity on neoadjuvant chemotherapy

Table 10 presents the distribution of randomly selected cases with non-heterogeneous tumors according to morphologic variant, focality, grade of differentiation and molecular subtype before and after NACHT performed, comparable to the heterogeneity cases from Table 2 / task 1

Table 9 Non- heterogeneous tumors

№	Age	Morphological variant based on assessment of the tumor (Biopsy Resectate and metastases)	Tumor focality based on overall assessment	ypT	ypN	G before neoadjuvant therapy	G after neoadjuvant therapy	Molecular subtype before neoadjuvant therapy	Molecular subtype after neoadjuvant therapy	Therapy	pT on Sataloff after neoadjuvant therapy	pN pT on Sataloff after neoadjuvant therapy
1	67	lobular	unifocal	2	2	3	3	2	1	Epirubicine+ Endoxan	2	2
2	68	NST	unifocal	2	1	2	2	2	2	Epirubicine+ Endoxan	2	3
3	38	NST	unifocal	Tis	0	3	Complete pathologic response	3	Complete pathologic response	Docetaxel + Herceptin- Perjeta	1	2
4	73	NST	unifocal	1b	1	3	3	3	4	Paclitaxel + Carboplatin	3	4
5	67	NST	unifocal	2	0	2	2	1	1	Epirubicine+ Endoxan	2	2
6	72	NST	unifocal	1a	0	3	2	3	2	Docetaxel + Herceptin- Perjeta	1	2
7	41	NST	unifocal	1c	0	3	3	2(HER2)	2	Docetaxel + Herceptin- Perjeta	3	2
8	34	NST	unifocal	Tis	0	3	Complete pathologic response	3	Complete pathologic response	Docetaxel + Herceptin- Perjeta	1	2
9	41	NST	unifocal	2	2	3	3	2HER2	3	Docetaxel + Herceptin- Perjeta	3	2
10	30	NST	unifocal	1c	1	2	2	2	2	Epirubicine+ Endoxan	3	4
11	33	NST	unifocal	0	0	3	Complete pathologic response	4	Complete pathologic response	Paclitaxel + Carboplatin	1	1
12	44	NST	unifocal	1	1	3	3	3	3	Docetaxel + Herceptin- Perjeta	3	3

Legend: G grade: 1- highly differentiated, 2- moderately differentiated, 3 -low differentiated

Molecular subtype: 1- luminal A, 2- luminal B, 2(HER2)- luminal HER2,

3- HER2 enriched, 4- TNCG

Sataloff score pT: 1- total (no residual tumor), 2- therapeutic effect > 50%, 3- therapeutic effect <

50%, 4- no therapeutic effect, pN: 1- therapeutic effect, no metastases, 2- no metastases, but no therapeutic effect, 3- therapeutic effect, metastatic lymph node, 4- metastases present without therapeutic effect

A review of the individual variables after NACHT was performed, showed the following:

3.1 Post-therapeutic T status:

Following the ypT of patients with a heterogeneous tumor:

- five of the cases are with T1
- two cases are with T2
- three cases are with T3
- two cases are with T4.

Corresponding to the group of non-heterogeneous tumors are:

- two cases are with Tis
- five cases are with T1
- four cases are with T2
- one patient has no residual Tumor - T0 after neoadjuvant therapy

Comparison of tumor volume and infiltration after NACHT (ypT) between tumors from the heterogeneity group and those without heterogeneity showed no statistically significant difference

$$\chi^2 = 10.480, \text{ df} = 6, \text{ p} = 0.1058$$

From the results obtained in our study, when determining the impact of neoadjuvant chemotherapy on breast cancer, it is striking that there was no reported complete pathological response in the group with pronounced tumor heterogeneity. Patients in this group also reported a significantly higher incidence of residual tumor after NACHT, which corresponds to data published in the scientific literature.

3.2 Post-therapeutic N status:

Following ypN in the group of patients with heterogeneous tumor the results obtained are as follows:

- N0 are two patients and one has micrometastasis
- three patients are in the pN1 group
- five are in the ypN2 group
- one is in the ypN3 group

Following ypN in the group of patients with non-heterogeneous tumor are as follows:

- six are in the ypN0 group
- four are in the ypN1 group
- two are in the ypN2 group

- Comparison of ypN status between tumors from the heterogeneous group and those without heterogeneity showed no statistically significant difference.

$$\chi^2 = 6.933, df = 6, p = 0.3270$$

Despite the large variation in tumor therapeutic response, the lack of a reported complete pathologic response in the group with marked tumor heterogeneity is striking. As well as more pronounced lymphatic involvement after performed NACHT in the heterogeneous group of patients.

3.3 Tumor differentiation

The tumor differentiation determined in the tumor components before neoadjuvant therapy based on morphological characteristics of NST (Non specified type) and/or lobular/mucinous component in the group of patients with heterogeneous tumor shows the following distribution:

G3 (low differentiated) are four patients

G2 (moderately differentiated) are six patients

G1 (highly differentiated) are two cases (of which one mucinous accepted as G1)

Matched tumor differentiation of the group of patients with non-heterogeneous tumor were as follows:

G3 (low differentiated) were nine cases

G2 (moderately differentiated) were three cases

Comparison of tumor differentiation in each of the two groups (before and after neoadjuvant chemotherapy) showed no statistically significant difference.

Comparison of tumor differentiation before and after neoadjuvant chemotherapy in heterogeneous tumors did not show a significant change in tumor grade.

Stuart-Maxwell $\chi^2 = 2.333, df = 2, p = 0.3114$ (Табл.10)

Table 10 Comparison of tumor differentiation before and after neoadjuvant chemotherapy in heterogeneous tumors

	G1 (before NACHT)	G2 (before NACHT)	G3 (before NACHT)
G1 (after NACHT)	0	0	0
G2 (after NACHT)	2	4	1
G3 (after NACHT)	0	2	3

Comparison of tumor differentiation before and after neoadjuvant chemotherapy in non-heterogeneous tumors did not show a significant change in tumor grade.

Stuart-Maxwell $\chi^2 = 1.000$, df (conservative) = 2, p = 0.607 (Table 11)

Table 11 Comparison of tumor differentiation before and after neoadjuvant chemotherapy in non-heterogeneous tumors

	G1 (before NACHT)	G2 (before NACHT)	G3 (before NACHT)
G1 (after NACHT)	0	0	0
G2 (after NACHT)	0	3	1
G3 (after NACHT)	0	0	5

Three cases were not compared because of a complete pathologic response in the resectate.

Comparison of the presence of a change in tumor differentiation in the two groups before neoadjuvant chemotherapy did not show a statistically significant difference

$\chi^2 = 2.250$, df = 1, p = 0.1336

Despite non-significant differences, the absence of highly differentiated G1 tumors in the non-heterogeneous tumor group was clearly evident. The presence of these in the group of patients with heterogeneous tumor is due to the NST component, which is high grade and was not affected by the neoadjuvant chemotherapy performed.

3.4 Post-therapeutic surrogate molecular status:

When comparing the surrogate molecular subtype of tumors after neoadjuvant chemotherapy in the group with marked heterogeneity, the following was found:

- Luminal A subtype are four cases
- Luminal B are seven cases, of which:
 - four are luminal B
 - three are HER 2 enriched subtype
- One of the cases is TNBC /triple negative breast carcinoma/

In the group of patients with non-heterogeneous tumors, the molecular subtype distribution after neoadjuvant chemotherapy was as follows:

- Luminal A was reported in two cases
- Luminal B are four cases,

- Two cases were HER2 positive
- One case is TNBC /triple negative breast carcinoma/
- Three cases have a complete pathological response

Three of the cases with non-heterogeneous tumors had a complete pathologic response after NACHT, two of which were HER2 positive and one was of the triple negative type. In five cases we have a change of the surrogate molecular subtype after neoadjuvant chemotherapy - in three cases a decreased in HER 2 expression after therapy was reported (expected effect), in one case we have a change from luminal B type to luminal A type, due to reduced proliferative activity (also expected effect after therapy) and in one case there is a loss of estrogen expression in HER 2 enriched subtype of luminal B.

When examining a resectat from heterogeneous tumors after neoadjuvant chemotherapy, the presence of two tumor components simultaneously existing in six of the cases was found, and in three of the cases they were of a different surrogate molecular subtype.

Overall, in the heterogeneous population, a surrogate molecular subtype change was observed in eight cases.

In the non-heterogeneous population, a change of the molecular subtype was observed in five cases affecting a change in the steroid and HER2 receptor status in four cases and the proliferative activity in one case.

Changes in molecular expression after NACHT are widely known in the literature published to date and are analogous to our results, which is the main reason for the re-evaluation of residual tumor after therapy or in case of relapse.

Overall, despite the trends reported, the comparison of molecular subtype change in cases in the two groups after neoadjuvant therapy did not show a statistically significant difference. The tendency to have a surrogate molecular subtype change in heterogeneous tumors is well marked, but in the small number of patients studied is insignificant statistically. $\chi^2 = 3.600$, $df = 1$, $p = 0.0577$

3.5 Therapeutic response:

When comparing heterogeneous and non-heterogeneous tumors according to response to primary tumor therapy - Sataloff ypT category was found:

For the Heterogeneous Group:

- Category 2 according to Sataloff are four cases in which we have more than 50% therapeutic response but less than a complete or near-complete therapeutic response (less than 5% residual tumor each)
- Category 3 according to Sataloff are six cases with less than 50% therapeutic response
- Category 4 according to Sataloff are two cases without data on therapeutic response.

In heterogeneous tumors, we have not reported a case with complete response of the primary tumor

For the Non-Heterogeneous Group:

- Category 1 according to Sataloff in the group of non-heterogeneous tumors are four cases with complete or almost complete pathological response.
- Category 2 according to Sataloff with more than 50% pathological response (incomplete) are three cases.
- Category 3 according to Sataloff with response less than 50% are five cases

In the group of non-heterogeneous tumors, we do not have a case without response of the primary tumor

When comparing response to therapy, reported according to Sataloff in the primary tumor in the two groups showed a statistically significant difference $\chi^2 = 14.400$, $df = 4$, $p = 0.0061$

When comparing the heterogeneous and non-heterogeneous tumors based on response to therapy according to Sataloff ypN category, we found:

In the group of heterogeneous tumors, the distribution is as follows:

- Sataloff category 2 is reported in one case in which neither metastases nor evidence of therapeutic effect are present.
- Sataloff category 3 is reported in five cases with data of therapeutic effect and the presence of residual metastases in the lymph nodes,
- Sataloff category 4 are five cases with no therapeutic effect in the metastatic component, and in one of them there was evidence of a necrotic NST component in the vital mucinous component of the tumor.

It must be noted that in the heterogeneous group, we have one case with therapeutic response without a metastatic component.

In the group of non-heterogeneous tumors, the distribution is as follows:

- Sataloff category 1 - one case with therapeutic response without a metastatic component.
- Sataloff category 2 - seven cases with no evidence of involvement or metastases.
- Sataloff category 3 - two cases with evidence of metastatic process (data of vital metastasis) and therapeutic response.
- Sataloff category 4 - two cases with no evidence of response to therapy in the metastatic component.

When comparing response to therapy, reported by Sataloff in axillary metastases in the two groups, no statistically significant difference was reported.

$$\chi^2 = 3.771, \text{ df} = 3, \text{ p} = 0.2872$$

From the results obtained in our study, when determining the impact of neoadjuvant chemotherapy on breast cancer, it is striking that there was no reported complete therapeutic impact in the group with pronounced tumor heterogeneity. Patients in this group also reported a significantly higher incidence of residual tumor and lymphatic involvement after NACHT, ypN, but with a higher degree of differentiation. In heterogeneous tumors, the possibility of simultaneous presence of therapeutically resistant and sensitive cell populations within a single lesion determines the possibility of development of therapeutic resistance and persistence of minimal residual disease. This dependence is expected to shorten the progression-free survival, increase the risk of relapse and worsen the overall prognosis.

However, looking at our results, although initially there was a tendency towards a complicated course and resistance to therapy in the heterogeneous tumors, after the statistical assessment, the differences in the therapeutic response between the two groups remained non-significant. Similar data was published by Yates L.R et al, 2015, who did not report differences in the response to NACHT in heterogeneous breast tumors.

Figure 5 and Figure 6 graphically present the histological findings of two cases from the whole population studied, one with a complete pathological response and one with an incomplete (partial) pathological response.

Example of complete pathologic response in high-grade breast carcinoma, post-therapy status and quadruple negative status for ER, PgR, AR and HER 2. Ki 67 determined on biopsy as 60%. Before neoadjuvant therapy, the tumor had the following histological appearance (Fig. 5.1)

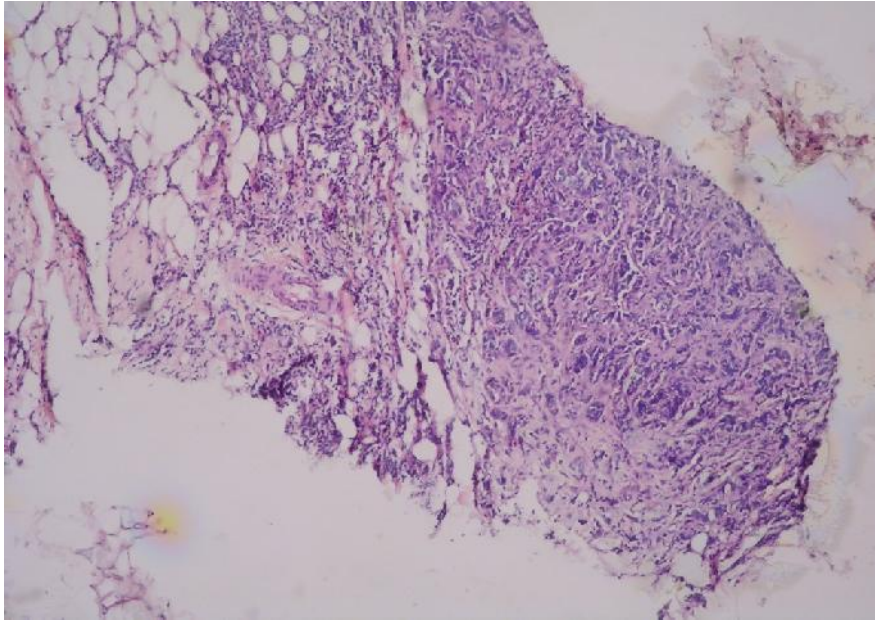


Figure 5.1 Primary tumor – high-grade NST ductal carcinoma G2-3 (HE, 40x)

In the resectate following the operation after the neoadjuvant therapy, no residual tumor is found (Fig. 5.2)

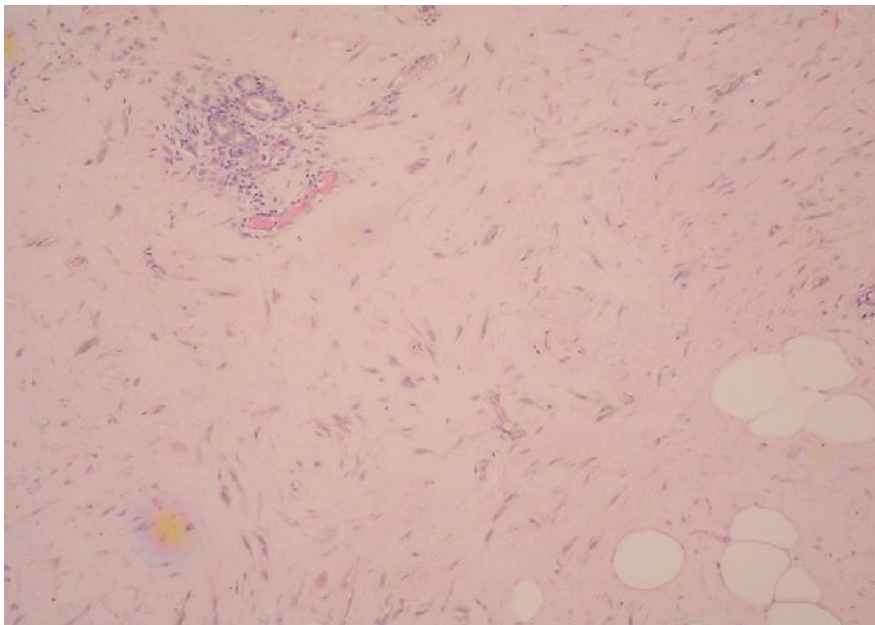


Figure 5.2 Breast parenchyma with atrophic terminal ductolobular units, cellular fibrous stroma in the tumor bed. (HE, 200x)

An example of incomplete pathological response in a patient with G2 NST ductal carcinoma proven on needle biopsy (Fig. 6.1) and ER (Fig. 6.2), HER2 (Fig. 6.3) and Ki-67 (Fig. 6.4) status, respectively

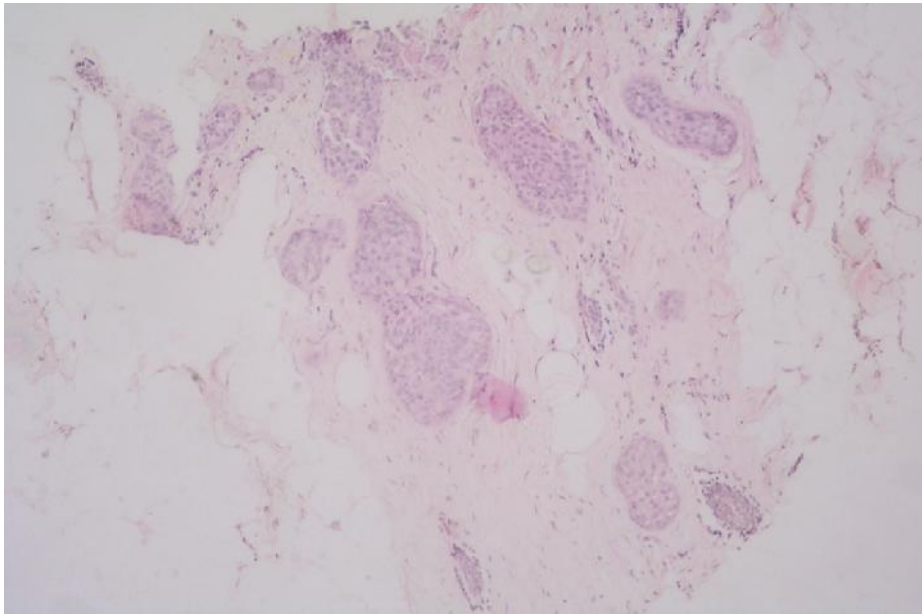


Figure 6.1 Preoperative biopsy invasive NST (ductal) carcinoma G2- groups of tumor cells and structures characteristic of DCIS -G2 (20x HE)

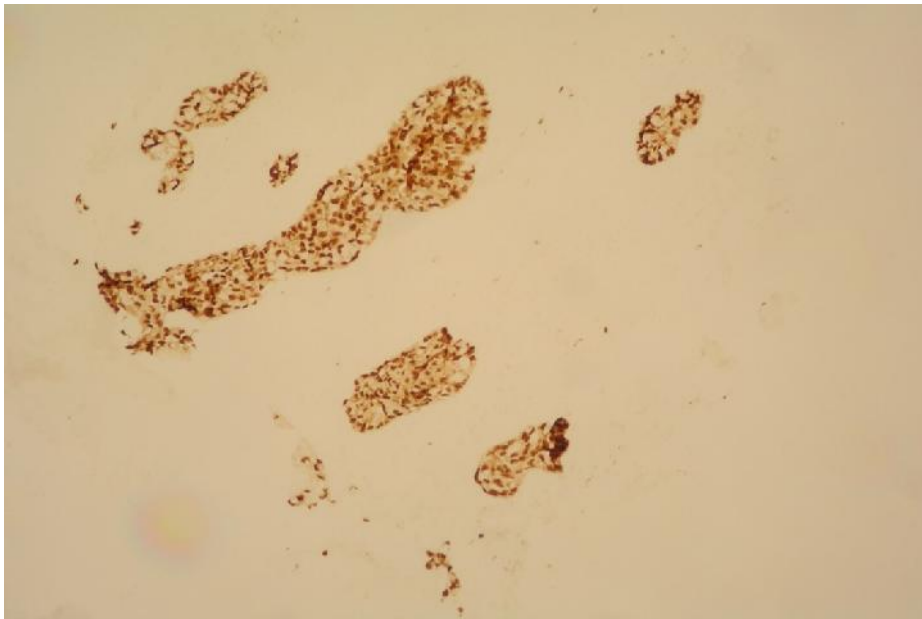


Figure 6.2 After immunohistochemical examination – ER+ status moderate intensity in more than 80% of tumor cells (20x ER). PgR expression in the patient's tumor cells was weak and in a small percentage of cells

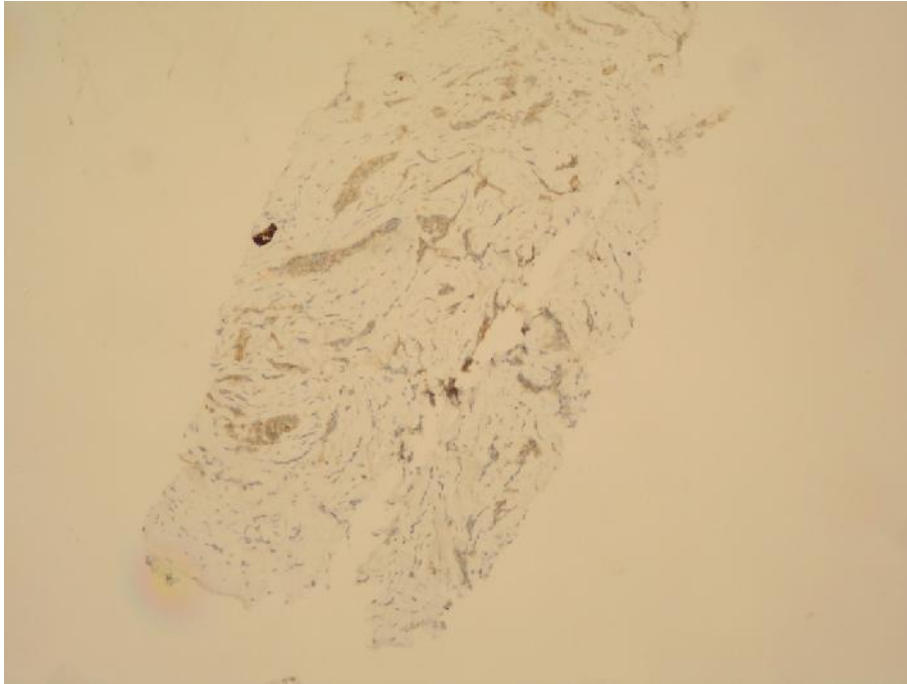


Figure 6.3 HER 2 2+ Weak to moderate circumferential staining in more than 10% of tumor cells (20x HER 2). The case was verified as ISH negative

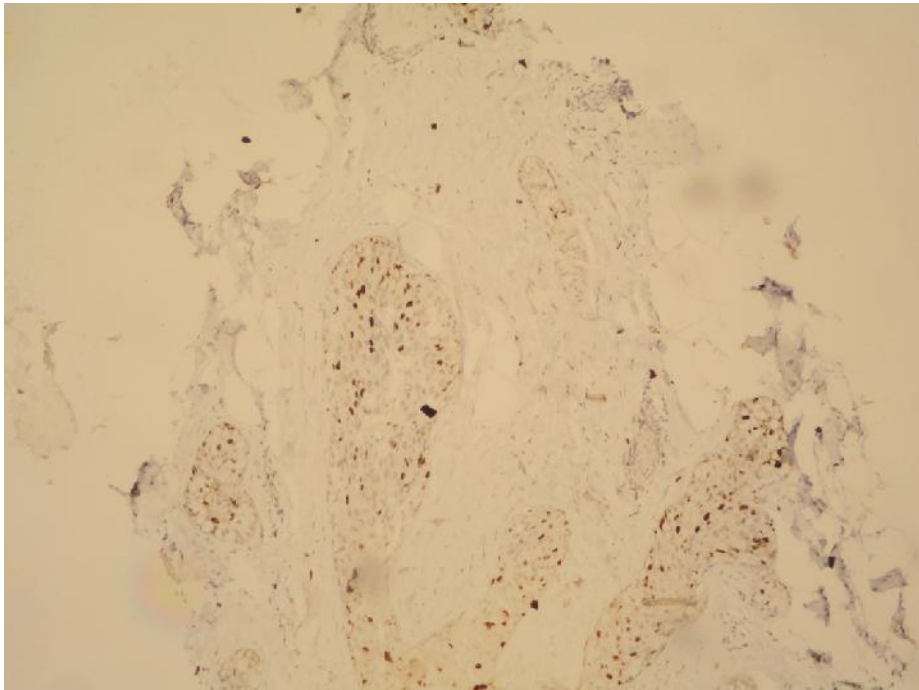


Figure 6.4 Ki 67 – 25% of tumor cell nuclei are positive for the marker (20 x Ki 67)

In the resectate following the operation after neoadjuvant therapy, a residual tumor is found in a small area (Fig. 8.5)

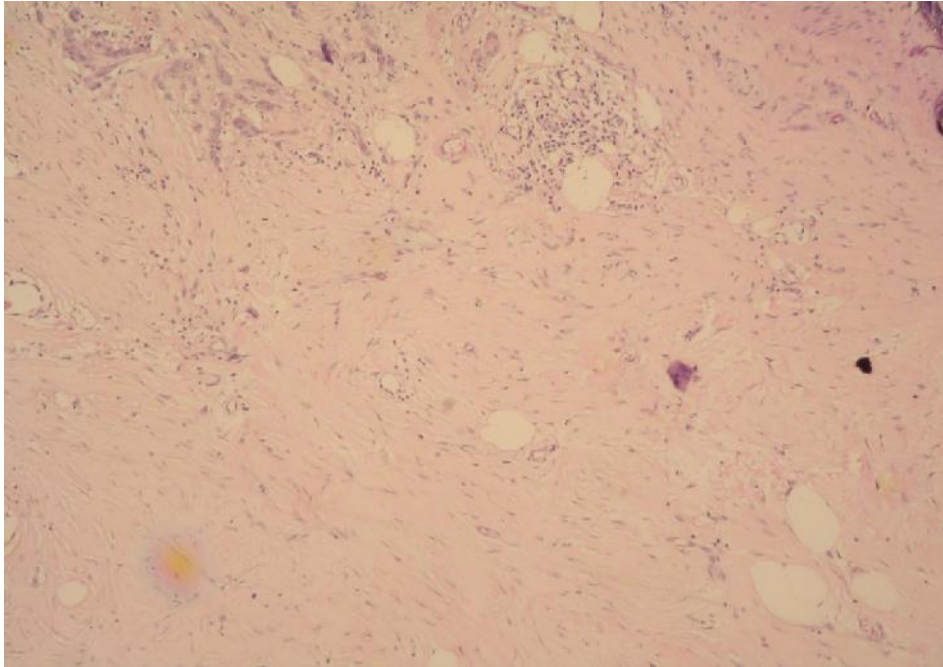


Figure 6.5 Post-therapy, foci of cellular fibrosis were noted in the tumor bed, among which groups of tumor cells were found (up to about 15% of the area of the tumor bed in the examined sections). Small accumulations of lymphocytes and microcalcifications are observed (HE, 100x).

4. Task 4: To determine recurrence and survival in patients with proven tumor heterogeneity and neoadjuvant chemotherapy.

The number of cases studied is too small to make general conclusions in practice, but at the same time they show some trends that can serve as a guideline for future studies.

In our study, in two of the cases with tumor heterogeneity a disease progression was detected, one with liver metastases (the patient survived 5 years), and the other with bone metastases (the patient died).

Respectively, in two of the cases without tumor heterogeneity, disease progression was detected, one with lung metastases (the patient died) and the other with pleural metastases (the patient died). The remaining patients who died were at an advanced stage with persistent disease.

No difference in overall survival was found between the group of patients with heterogeneous tumors and patients with tumors without marked heterogeneity.

Log-rank test $\chi^2=0.0981347$; Df=1; p= 0.754080. (Fig. 7)

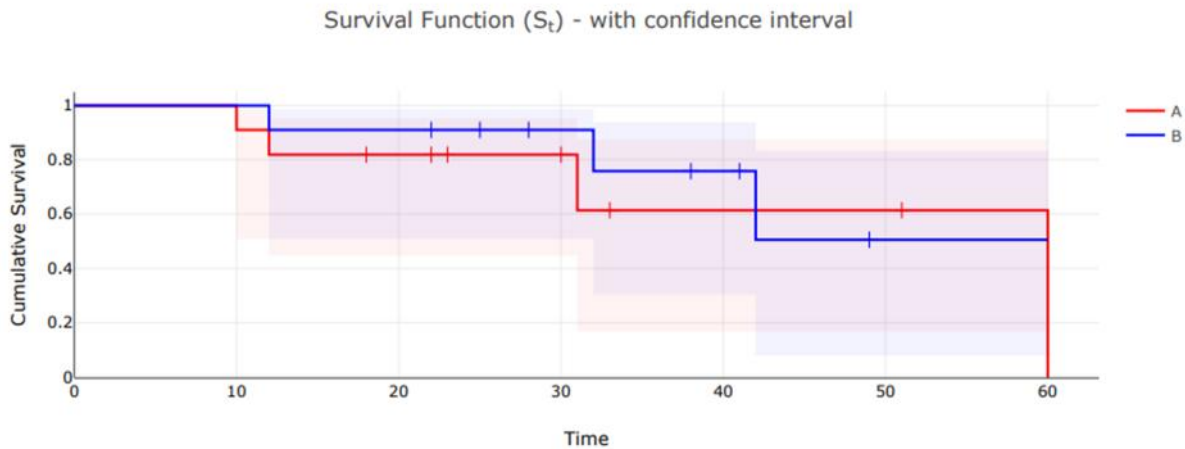


Figure 7 Kaplan-Meier curves showing overall survival in the group of patients with heterogeneous tumors (A) and patients with tumors without marked heterogeneity (B).

No difference in disease-free survival was found between the group of patients with heterogeneous tumors and patients with tumors without marked heterogeneity

Log-rank test $\chi^2 = 0.00279535$; Df=1; $p = 0.957835$. (Fig. 8)

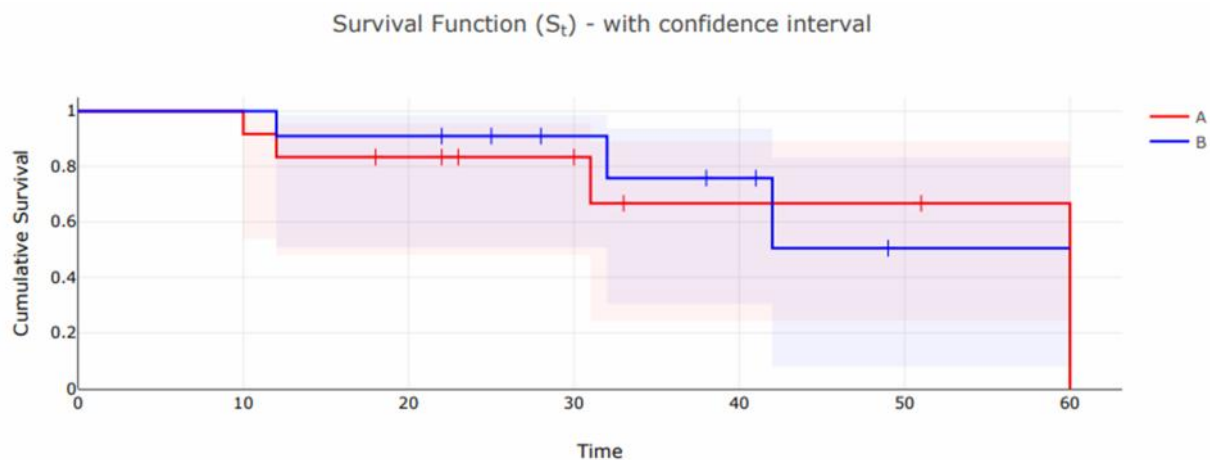


Figure 8 Kaplan-Meier curves showing disease-free survival in the group of patients with heterogeneous tumors (A) and patients with tumors without marked heterogeneity (B).

Despite the small number of patients studied and analyzed, the lack of significant influence of tumor heterogeneity on overall survival and disease-free survival is reported. We assume that the essence of tumor heterogeneity, the morphological and molecular type of tumor components are

decisive in a clinical sense. If both tumor components can be targeted or influenced with a specific neoadjuvant therapy, the presence of heterogeneity does not change the clinical behavior and evolution. If one component is low-grade, steroid receptor-positive, with a good clinical prognosis, and the other is high-grade, sensitive to targeted therapy or chemotherapy, then neoadjuvant therapy would affect only the high-grade, aggressive component, and would not be expected to affect the indolent tubular component leading to tumor persistence. Such changes in tumor biology determine persistence in short term, but are not expected to result in an event significantly worsening overall prognosis and survival. Our results support the data in literature, that tumor heterogeneity is a leading factor in persistence, and only under certain conditions can become a major factor in tumor progression as well. / summarized analysis by Dzobo K et al, 2018/

No difference in overall survival was found between the group of patients with ypT in situ and ypT1 tumors versus those with ypT 2-4

Log-rank test $\chi^2 = 0.477286$; Df=1; p= 0.489654. (Fig.9)

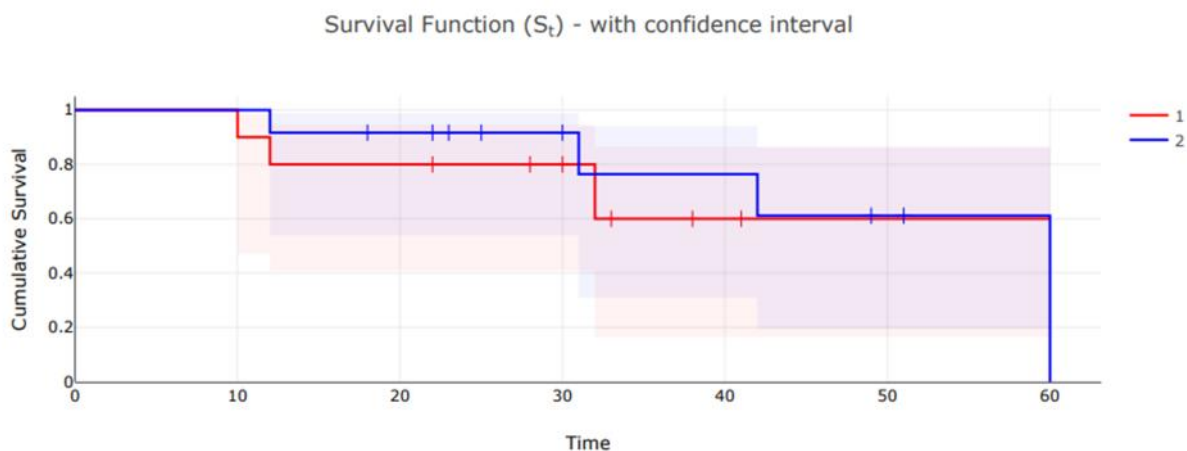


Figure 9 Kaplan-Meier curves showing overall survival in the group of patients with ypT in situ and ypT1 (A) and those with ypT 2-4 (B).

No difference in disease-free survival was found between the group of patients with ypT in situ and ypT1 tumors versus those with ypT 2-4

Log-rank test $\chi^2 = 0.00279535$; Df=1; p=0.957835. (фиг. 10)

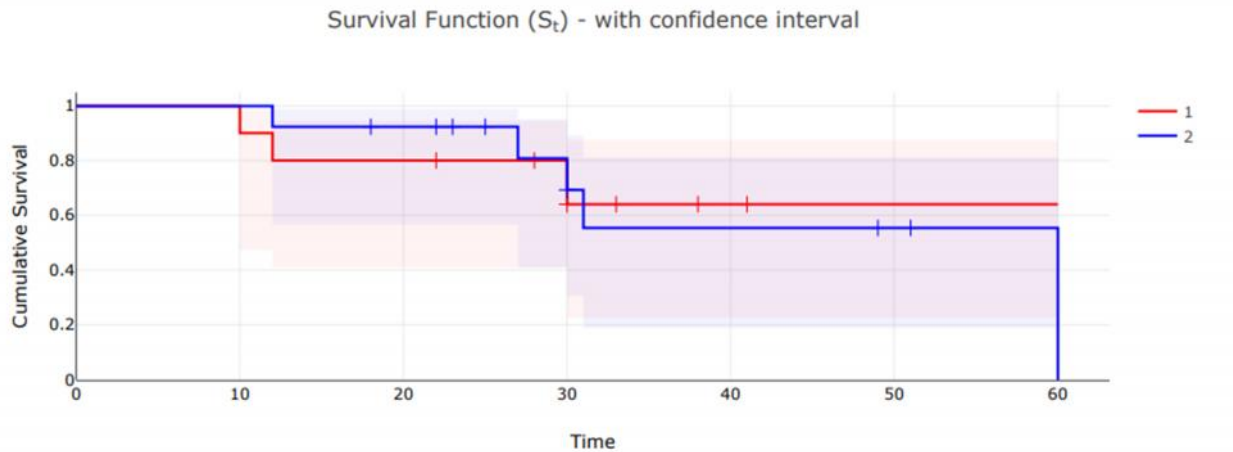


Figure 10 Kaplan-Meier curves showing disease-free survival in the group of patients with ypT in situ and ypT 1 (A) versus those with ypT 2-4 (B).

No statistically significant difference in overall survival was found between the group of patients with tumors and ypN positive status and ypN negative status

Log-rank test $\chi^2 = 3.746566$; Df=1; p= 0.0529161 (Fig.11)

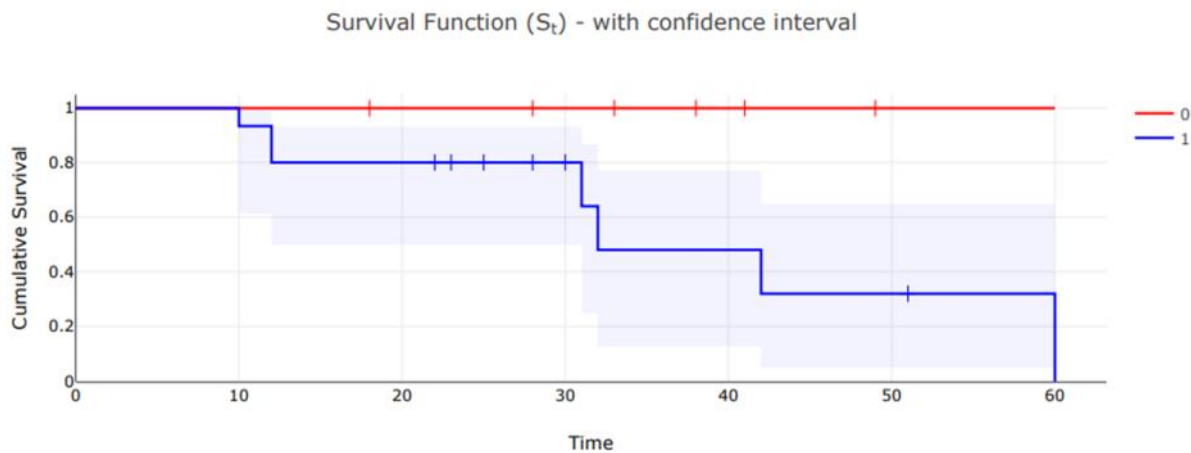


Figure 11 Kaplan-Meier curves showing overall survival in the group of patients with ypN negative status (A) and patients with ypN positive status (B).

A significant difference in disease-free survival was found between the group of patients with tumors and ypN positive status and ypN negative status

Log-rank test $\chi^2 = 4.305432$; Df=1; p= 0.0379908 (Fig.12)

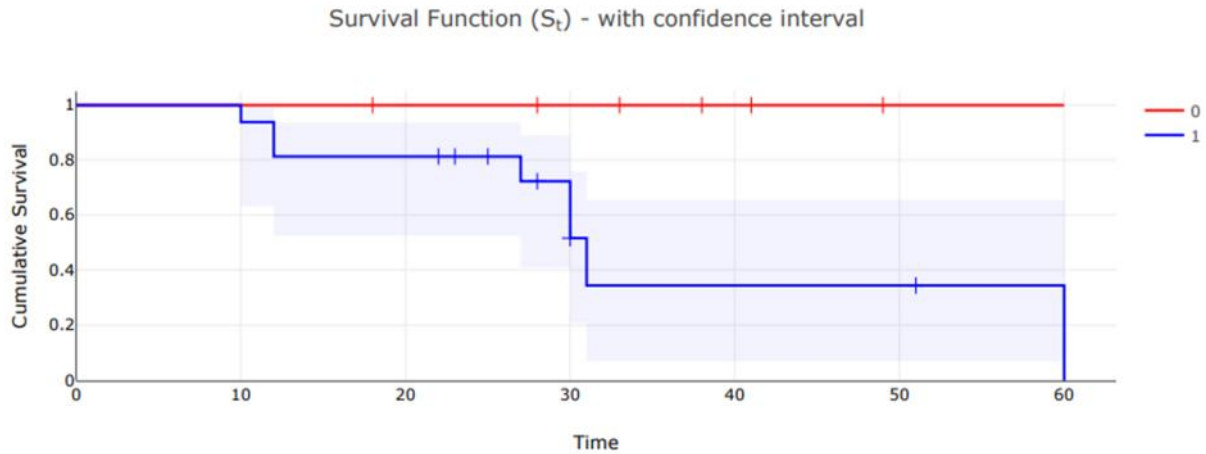


Figure 12 Kaplan-Meier curves showing disease-free survival in the group of patients with ypN negative status (A) and patients with ypN positive status (B).

No difference in overall survival was found between the group of patients with a complete/near-complete pathological response and patients with residual tumor, with varying degree of response to no response

Log-rank test $\chi^2 = 1.227413$; Df=1; p= 0.267911 (Fig. 13)

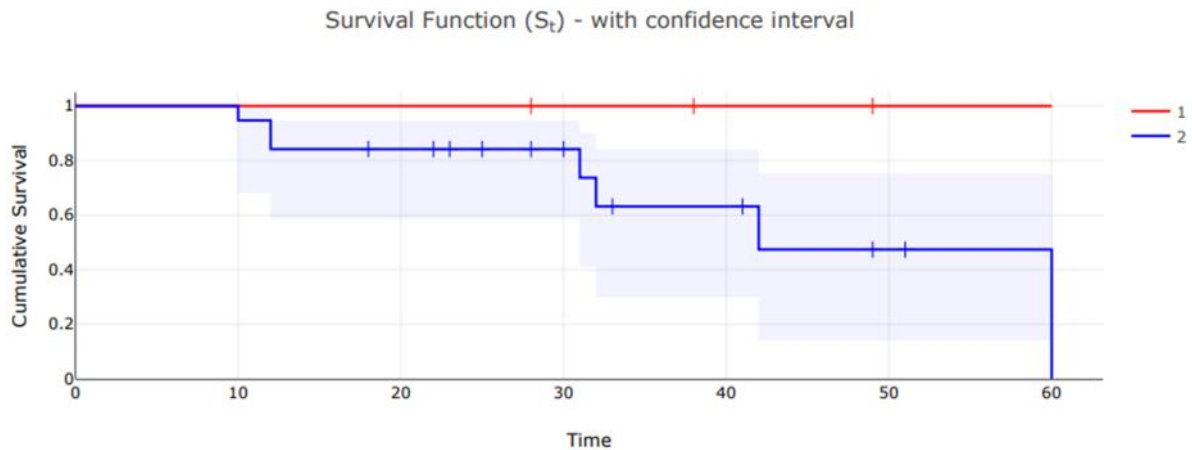


Figure 13 Kaplan-Meier curves showing overall survival in the group of patients with a complete/near-complete pathological response (A) and patients with residual tumor with varying degree of response to no response (B).

No difference in disease-free survival was found between the group of patients with a complete/near-complete pathological response and patients with residual tumor, with varying degree of response to no response.

Log-rank test $\chi^2 = 1.393110$; Df=1; p= 0.237881 (Fig. 14)

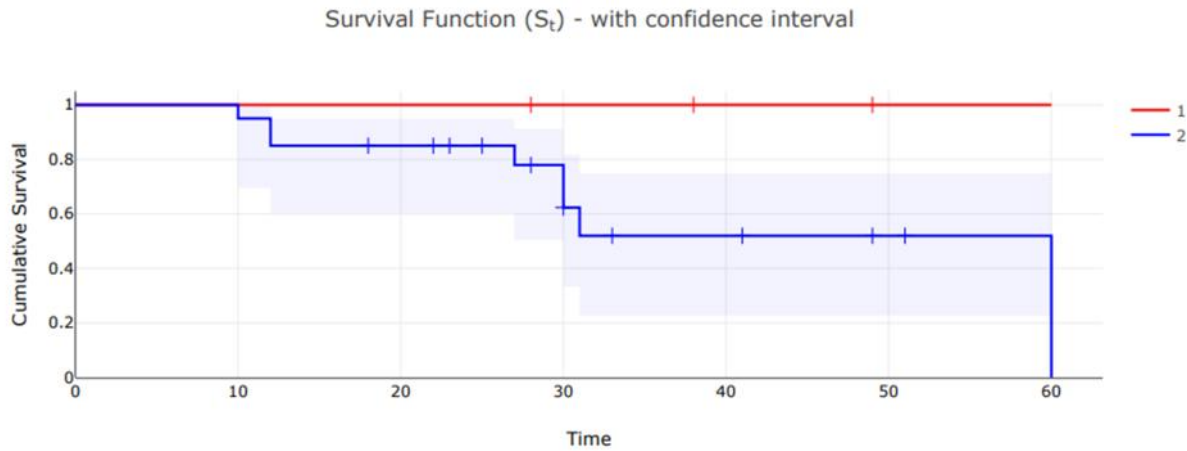


Figure 14 Kaplan-Meier curves showing disease-free survival in the group of patients with complete/near-complete pathological response (A) and patients with residual tumor with varying degree of response to no response (B).

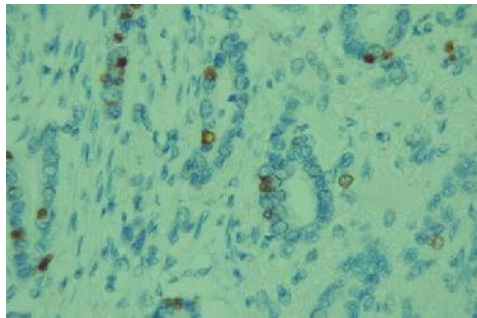
In our study, survival by grade and molecular subtype was not tracked because heterogeneous tumors are difficult to stratify.

From the results described above, although not significant, there was a tendency for the negative post-treatment TNM status to be associated with a better prognosis. Primarily this applies to the negative nodal status after neoadjuvant therapy which shows, although statistically insignificant, a tendency to be associated with better overall survival compared to cases with positive nodal status. Another indicator with a distinct trend for a difference in survival, although not significant, showed a ypT response of the primary tumor to neoadjuvant therapy, reported according to Sataloff. The results we present confirm the tumor volume and nodal status as long-established indicators of independent prognostic value, part of the Nottingham prognostic index successfully used to stratify breast cancer patients since 1973, and also being associated with poor prognosis. Despite the small number of patients studied and analyzed, the lack of significant influence of tumor heterogeneity on overall survival and disease-free survival is reported. The data published to date in literature, confirm the idea that heterogeneous tumors are more therapeutically resistant without significantly affecting overall survival, which is compatible with our results.

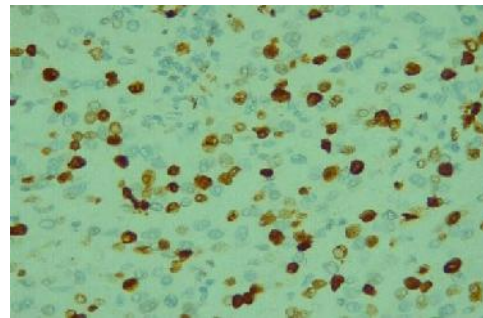
5. Task 5: To determine the role of proliferative and apoptotic activity before and after neoadjuvant chemotherapy in a tumor population containing cases with tumor heterogeneity

The reported mean Ki-67 in the study population was 26% (ranging from 8% to 94%).

Examples of Ki-67 values lower than 14% and equal or higher than 14% are presented in Fig. 15.



A

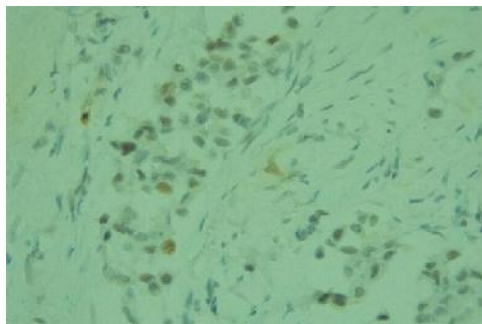


B

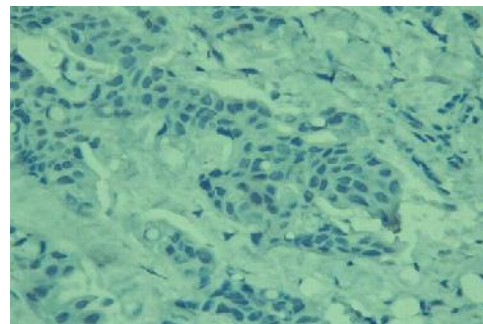
Figure 15. A. Ki-67 values of less than 14%, Ki-67, 400x; B. Ki-67 values equal to or above 14% Ki-67, 400x

Caspase-3 was reported positive in 17 of the cases and negative in 88.

Examples of caspase-3 immunohistochemical positivity and negativity are presented in Figure 16.



A



B

Фигура 16 A. Caspase-3 позитивна, caspase-3, 400x; B. Caspase-3 негативна, caspase-3, 400x

In the study population, caspase-3 negative cases had a measured Ki-67 activity at average of 25% (ranging from 8% to 94%). Cases positive for caspase-3 demonstrated average Ki-67 values of 35% (ranging between 12% and 87%). A similar parallel growth of apoptosis and proliferation, with predominance of proliferative activity, has been described in the literature in tumor progression with high turnover and confirms the theory of apoptosis-induced proliferation in malignant processes.

Comparing the values of the two markers expressed in the two groups did not show a statistically significant difference. K-W=1.7631, p=0.1842.

5.1 Ki-67 relationships with the investigated prognostic markers

Comparing with the studied prognostic parameters, Ki-67 demonstrated the following relationships:

A statistically significant difference was found between the Ki-67 index (on diagnostic biopsy) and the degree of differentiation (G) K-W = 24.2932, p<0.0001.

As expected, highly differentiated tumors (G1) express the lowest values of Ki-67 index and vice versa poorly differentiated (G3) tumors the highest values of Ki-67 index (Table 12)

Table 12 Ki-67 levels (on diagnostic biopsy) and degree of differentiation (G)

Grade	Count	Median	Min	Max
G1	2	12	10	14
G2	47	20	8	73
G3	54	37	11	94

A statistically significant difference was reported between Ki-67 expression (on diagnostic biopsy) and tumor (surrogate) molecular subtype (on diagnostic biopsy)

K-W = 28.5439, p< 0.00000967538.

Luminal A carcinomas presented with the lowest Ki-67% index compared to the other categories. (Table 13)

Table 13 Ki-67 level (on diagnostic biopsy) and (surrogate) tumor molecular subtype (on diagnostic biopsy)

Molecular subtype	Count	Median	Minimum	Maximum
Luminal A	15	14	9	20
Luminal B Ki-67 ≥14%	45	35	8	94
Luminal B HER2 positive	7	25	19	43
HER 2	21	35	14	61
TNT	9	27	18	87

A statistically significant difference was found between levels of Ki-67 (on diagnostic biopsy) and the volume and infiltration of the primary tumor after NACHT, on resection.

K-W = 11.7944, $p < 0.0377169$.

Tumors demonstrating complete pathological response (referred to yT0 and yTis) were among the tumors with the highest Ki-67 index among the studied group. (Table 14)

Table 14 Ki-67 (on diagnostic biopsy) and pathologic response to NACHT examined on resection specimen

ypT	Count	Median	Minimum	Maximum
0	12	37.5	14	87
1	22	25.5	9	70
2	47	23	8	94
3	8	35	14	90
4	12	21,5	10	73
In situ (ypTis)	4	48	40	60

No statistically significant difference was found between levels of Ki-67 expression (on diagnostic biopsy) and extent of regional lymph node involvement after NACHT on resection specimen

K-W = 4.19294, $p > 0.241366$ (Table 15)

Table 15 Ki-67 levels (on diagnostic biopsy) and level of regional lymph node involvement (based on post-therapy resection specimen)

ypN	Count	Median	Minimum	Maximum
0	32	27.5	11	70
1	31	27	9	94
2	26	25.5	10	87
3	13	20	8	59

No statistically significant difference was found between Ki-67 levels (on diagnostic biopsy) and the effect of NACHT reported according to Sataloff category T

K-W = 3.2385, $p = 0.0719229$ (Table 16)

Table 16 Ki-67 levels (on diagnostic biopsy) and the effect of NACHT reported according to Sataloff category T

Sataloff T	Count	Median	Minimum	Maximum
A (complete response)	18	40	14	61
B, C, D (non-complete response and no response)	71	26	9	94

No statistically significant difference was found between Ki67 levels (on diagnostic biopsy) and the effect of NACHT reported according to Sataloff category N

K-W = 1.40937 p> 0.703338 (Таблица 17)

Table 17 Ki-67 levels (on diagnostic biopsy) the effect of NACHT reported according to Sataloff category N

Sataloff N	Count	Median	Minimum	Maximum
A	8	39	18	60
B	22	26	14	70
C	32	26	9	87
D	23	35	9	94

5.2 Caspase-3 dependences with the studied prognostic markers

When comparing the investigated prognostic factors and parameters, caspase-3 demonstrated the following dependences:

A trend toward an association of caspase-3 (on diagnostic biopsy) with the primary tumor volume and infiltration after NACHT and surgery was reported, but the patient number was too small, making statistical significance largely implausible

$\chi^2 = 27.02$, Df=5, p=0. 0001 (Table 18)

Table 18 Expression of caspase-3 (on diagnostic biopsy) in relation to volume and infiltration of the primary tumor after NACHT and operative intervention

ypT	0	1	2	3	4	In situ
Caspase-3 negative	9 (8.57%)	17 (16.19%)	42 (40.00%)	8 (7.62%)	12 (11.43%)	0 (0.00%)
Caspase-3 positive	3 (2.86%)	5 (4.76%)	5 (4.76%)	0 (0.00%)	0 (0.00%)	4 (3.81%)

A statistically significant association was reported between caspase-3 expression (on diagnostic biopsy) and therapeutic effect after NACHT reported according to Sataloff category T.

$\chi^2 = 5.97$, Df=1, p=0. 0145.

More than 1/3 of patients with complete pathologic response after NACHT were caspase-3 positive on diagnostic biopsy, compared to only 1/8 expressing caspase-3 of those with incomplete or absent therapeutic response. (Table 19)

Table19 Caspase-3 expression (on diagnostic biopsy) associated with therapeutic effect after NACHT reported according to Sataloff T classification

Sataloff T	A (complete response)	B, C, D (non-complete response and no response)
Caspase-3 negative	11 (12.36%)	63 (70,79%)
Caspase-3 positive	7 (7.87%)	8 (8.99%)

No statistically significant relationship was found between caspase-3 expression (on diagnostic biopsy) and the degree of differentiation

$$\chi^2 = 0.64, \text{Df}=2, p=0.7251 \text{ (Table20)}$$

Table 20 Caspase-3 (on diagnostic biopsy) and degree of differentiation

Grade	G1	G2	G3
Caspase-3 negative	2 (1.94%)	40 (38.83%)	44 (42.72%)
Caspase-3 positive	0 (0.00%)	7 (6.80%)	10 (9.71%)

No statistically significant association was found between caspase-3 expression (on diagnostic biopsy) and ypN status $\chi^2 = 2.7, \text{Df}=3, p=0.4401$ (Table 21)

Table 21 Expression of caspase-3 (on diagnostic biopsy) and ypN status

ypN	N0	N1	N2	N3
Caspase-3 negative	24 (23.53%)	27 (26.47%)	22 (21.57%)	12 (11.76%)
Caspase-3 positive	8 (7.84%)	4 (3.92%)	4 (3.92%)	1 (0.98%)

No statistically significant association was reported between caspase-3 expression (on diagnostic biopsy) and therapeutic response reported by Sataloff category N

$$\chi^2 = 3.88, \text{Df}=3, p=0.2746 \text{ (Table 22)}$$

Table 22 Caspase-3 (on diagnostic biopsy) and reported response according to Sataloff category N

Sataloff N	A	B	C	D
Caspase-3 negative	6 (7.06%)	17 (20.00%)	25 (29.41%)	22 (25.88%)
Caspase-3 positive	2 (2.35%)	5 (5.88%)	7 (8.24%)	1 (1.18%)

No statistically significant association was reported between caspase-3 expression (on diagnostic biopsy) and molecular (surrogate) tumor subtype.

$$\chi^2 = 6.98, \text{Df}=4, p=0.1372 \text{ (Table 23)}$$

Table 23 Caspase-3 (on diagnostic biopsy) and molecular (surrogate) tumor subtype

Molecular subtype	Luminal A	Luminal B Ki-67 $\geq 14\%$	Luminal B HER2 positive	HER2	TNBC
Caspase-3 negative	12 (12.37%)	40 (41.24%)	7 (7.22%)	14 (14.43%)	8 (8.25%)
Caspase-3 positive	3 (3.09%)	5 (5.15%)	0 (0.00%)	7 (7.22%)	1 (1.03%)

In our study, although insignificant Caspase-3 and Ki-67 index reported on diagnostic biopsy can be related to the main prognostic factors - degree of differentiation, T and N status, while data described in the literature remain controversial.

High levels of caspase-3 trended toward a better response to NACHT, but not related to overall survival. Thus they could complement the predictive value of the proliferative marker Ki-67 in the assessment of therapeutic effect and in determining subsequent therapeutic behavior for patients who did not achieve a complete pathological response after NACHT.

As no statistically significant relationship was reported between therapeutic effect of NACHT and expression of caspase-3 or proliferative index Ki-67 in the context of different molecular subtypes, it is difficult to assess therapeutic response in luminal A, luminal B and triple-negative carcinoma based solely on expression of both markers / Ki-67 and/or activated caspase-3/. This contradicts the ideas in the oncology community to introduce a stand-alone Ki-67-based follow-up of therapeutic effect after NACHT in advanced hormone-positive breast carcinomas.

The results of our study on Ki-67 and caspase-3, in addition to everything mentioned above, show that the presence of more than 10% heterogeneous tumors (classified according to the component with a worse clinical prognosis) in a randomly selected population does not lead to significant deviations in the validity of the results and conclusions presented by other studies. This of course does not preclude the presence of a similar percentage of unaccounted heterogeneity in the samples described in these studies.

VII. Conclusions

- A frequency of heterogeneous tumors among a random sample of patients diagnosed with breast cancer was reported - 10.91%, with only 2% referred to molecular heterogeneity. Up to 90% of cases are proven on resectate. There was no proven statistically significant relationship between the number of tissue cylinders and the frequency of reported heterogeneity on CNB.
- The group of heterogeneous tumors is characterized by a significantly younger age, a tendency towards a larger tumor volume, a lower degree of differentiation, a higher frequency of multifocality or multicentricity, a higher frequency of primary resistance and recurrence, as well as the frequency of a second primary tumor.
- After neoadjuvant chemotherapy or target therapy, a change in the surrogate molecular subtype was reported in more than 50% of the examined patients, and in nearly 10% of the cases a second tumor component with a different molecular expression, requiring a different therapeutic approach, was detected. Changes are significantly more frequent in the heterogeneous population in the direction of loss of receptor expression.
- Cases of molecular heterogeneity, in which luminal type A breast carcinoma is combined with HER2-positive or triple-negative carcinoma, remain problematic, making it difficult to assign target therapy, with the impossibility of predicting therapeutic effect and long-term benefits.
- After neoadjuvant chemotherapy or target therapy, patients with proven intratumoral heterogeneity have a higher rate of incomplete or absent pathological response, which is subsequently associated with cancer progression, higher rates of recurrence and metastasis, but without shortened overall survival.
- The examination of Caspase-3 and Ki-67 expression in our study supported the currently available data in literature on the presence of apoptosis-induced proliferation, occurring to the greatest extent in the group of heterogeneous tumors, remaining a predictor for more severe course and worse prognosis.
- High levels of Caspase-3 at baseline biopsy are more informative than those of Ki-67, suggesting a better effect of NACHT, but worse overall prognosis for patients. But unlike Ki-67, Caspase-3

did not show a statistically significant association with certain molecular subtypes, histological grade or T-category after NACHT.

- After neoadjuvant chemotherapy or target therapy, negative nodal status /ypN/ and primary tumor response /ypT/ were indicative (although statistically insignificant) towards better disease-free survival and overall survival in both groups.

- Patients in the non-heterogeneous group showed a trend towards better progression-free survival associated with complete pathological response, but with no statistically significant difference with the heterogeneous group.

- There was also no statistically significant difference in overall survival between the group of patients with heterogeneous tumors and patients with tumors without marked heterogeneity.

VIII. Contributions of a scientific and original character

- The conducted research improves the understanding of intratumoral heterogeneity in breast cancer patients.

- The conducted study is the first complex study of patients with manifested morphological and/or molecular heterogeneity (based on the routinely used in practice surrogate markers ER, PgR, HER2, Ki-67), based on standard examinations of hospitalized patients at University Hospital "Dr. Georgi Stranski" – Pleven

- The conducted study add scientific data on the incidence of morphological and/or molecular heterogeneity and its impact on tumor evolution in a standard therapeutic approach

- Detailed information was gathered and the significance of standard prognostic variables included in the Nottingham prognostic index, combined with morphological and molecular features in the often problematic group of heterogeneous tumors, is reported.

- Caspase-3 expression was investigated as a marker with added informative value to the highly variable and uncertain prognostic and evaluative proliferative marker - Ki-67

- An attempt was made to comprehensively assess the complex and multifactorial influence of morphological and/or molecular heterogeneity and its impact on evolution, prognosis and therapeutic effect in patients with breast cancer.

IX. Contributions of practical value:

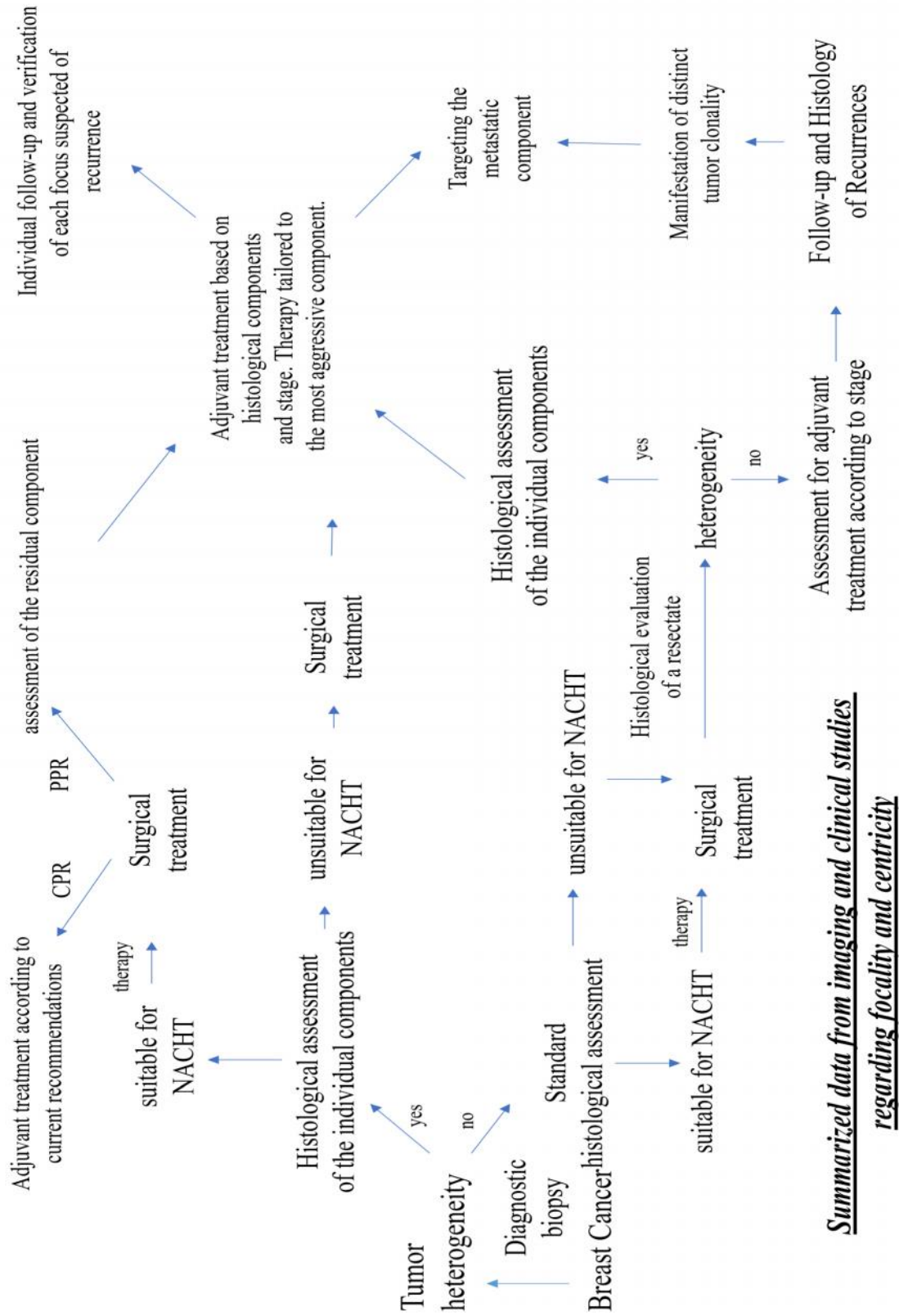
- A specific incidence of heterogeneous tumors in the general population of breast cancer patients for the Bulgarian population treated at the University Hospital "G. Stranski" – Pleven is reported.
- A low incidence has been reported in the general population of heterogeneous tumors proven on CNB compared to that reported on excisional biopsy.
- The relationship between tumors with manifested morphological and/or molecular heterogeneity and factors with low prognostic value - large tumor volume, low differentiation and positive nodal status - has been encountered
- Specific markers in the face of Ki-67 in combination with Caspase-3 relevant to follow-up of therapeutic effect and tumor evolution in breast cancer have been reported.
- According to the data we presented, the need to reassess HER2 status after NACHT remains controversial in HER2-positive patients, due to the lack of a component to warrant another therapeutic approach in cases without a complete response. There are no long-term benefits of changing the therapeutic regimen based on the type and frequency of HER2 conversion in relapse and metastasis as described.
- Data has been collected, justifying a general retesting in dynamics of the main morphological characteristics and surrogate molecular biomarkers, in breast cancer with available intratumoral heterogeneity, after performed NACHT, accounting for missed indolent or changed during therapy component from baseline biopsy, implying a different prognostic assessment, therapy and follow-up.
- The obtained results allow the development/construction of a diagnostic and therapeutic algorithm for patients with heterogeneous breast cancer, which can be used as an element/basis for a complex approach in order to refine and individualize therapy for patients with proven morphological and/or molecular heterogeneity.

Publications and participation in scientific events in connection with the dissertation work:

Nanev V, Milev H, Dimitrova D, Naneva S, Strashilov S, Yordanov A, Mihailova M, Ivanova S, Karcheva M, Ivanov I. Evaluation of cleaved caspase-3 and Ki-67 index on diagnostic biopsy in response to neoadjuvant chemotherapy in the context of post-treatment tumour ypT stage, ypN stage, grade, and molecular subtype. *Prz Menopauzalny*. 2024 Mar;23(1):31-40. doi: 10.5114/pm.2024.136962. Epub 2024 Mar 28. PMID: 38690065; PMCID: PMC11056723. Scopus Q2, SJR 2023 0.628; Web of Science Q2, IF 2023 2,5

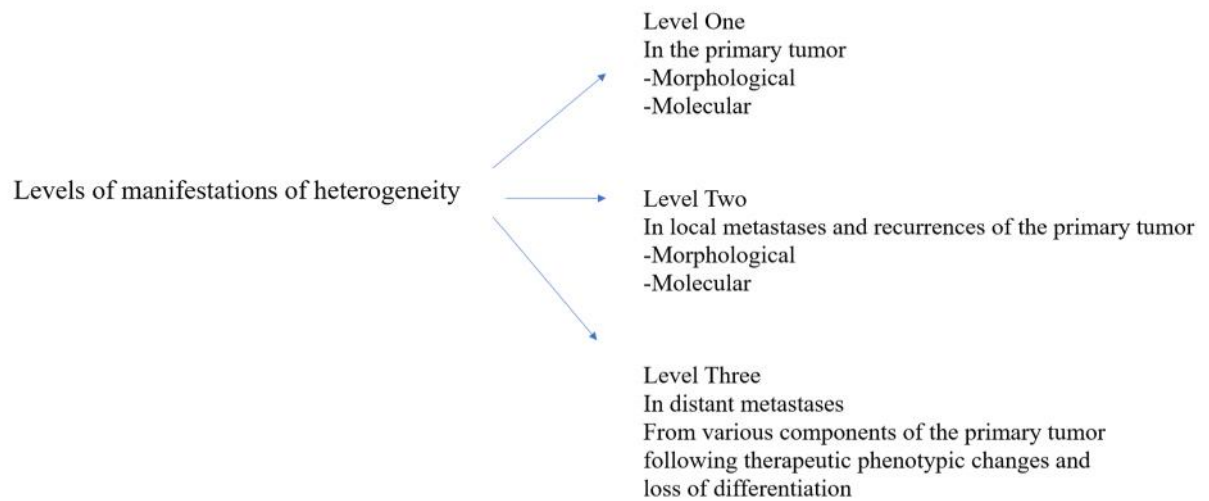
Nanev V, Naneva S, Yordanov A, Strashilov S, Ivanov I. (2022). Resistance to neoadjuvant chemotherapy in breast cancer with proven intratumoral heterogeneity: a clinical case. *Oncology in Clinical Practice*. 18. 134-138. 10.5603/OCP.2022.0015. Scopus Q4, SJR 2022 0.167; Web of Science Q4, IF 2022 0,5

Nanev V, Toneva L, Yordanov A, Strashilov S, Ivanov I. A case of non-palpable, multifocal, heterogeneous left breast carcinoma with mammographic presentation of clustered multiple microcalcifications. *Arch Balk Med Union*. 2020;55(3):489-494. <https://doi.org/10.31688/ABMU.2020.55.3.15> Scopus Q4, SJR 2020 0.145



Summarized data from imaging and clinical studies regarding focality and centrality

Appendix № 2



Appendix No. 3 Decision of the commission on ethics of scientific and research activity at the medical university - Pleven

Комисия по етика на научно-изследователската дейност КЕНИД Медицински университет - Плевен Ул. "Климент Охридски" № 1 Изх. <u>207-КЕНИД/03.06.2022</u> Телефон: 064 884 197	
РЕШЕНИЕ НА КОМИСИЯТА ПО ЕТИКА НА НАУЧНО-ИЗСЛЕДОВАТЕЛСКАТА ДЕЙНОСТ ПРИ МЕДИЦИНСКИ УНИВЕРСИТЕТ – ПЛЕВЕН	
Главен изследовател	доц. д-р Милена Карчева, дм
Изследователски център(ове)	➤ Катедра „Патологоанатомия“, Факултет Медицина, МУ-Плевен; ➤ Клиника по Онкологична Хирургия, УМБАЛ“Д-р Георги Странски“-Плевен
Заглавие на проекта	<i>„Оценка на експресията на апоптотичния фактор Caspase 3 и пролиферативен индекс оценен чрез Ki 67 преди и след неоадювантна химиотерапия при отделни молекулярни субтипове карцином на гърда в контекста на туморен хетерогенитет и анализ на тяхното клинично значение“</i>
Протокол № 67	
Получени, разгледани и одобрени от КЕНИД документи	✓ Форма-заявление за разглеждане и даване на решение за извършване на научни изследвания върху човешки същества; ✓ План-проект на научното изследване; ✓ Изследователски фиш на пациента ✓ Декларация за информирано съгласие на пациенте; ✓ Автобиографии на изследователския екип; ✓ Разрешения от ръководителите на изследователските центрове
Комисията по етика на научно-изследователската дейност при Медицински Университет - Плевен реши да разреши провеждането на научно изследване на тема: <i>„Оценка на експресията на апоптотичния фактор Caspase 3 и пролиферативен индекс оценен чрез Ki 67 преди и след неоадювантна химиотерапия при отделни молекулярни субтипове карцином на гърда в контекста на туморен хетерогенитет и анализ на тяхното клинично значение“</i> с главен изследовател доц. д-р Милена Карчева, дм, без периодичен надзор.	
юни 2022 г. гр. Плевен	Председател на КЕНИД: (проф. д-р С. Александър Янчевски, д.м.н.) Секретар на КЕНИД: (доц. Макрета Драганова)

Appendix No. 4

Clinical case	Focal	One/two-sided	Synchronous/metachronous	Histological variants	Therapeutic approach	Survivability
1	multifocal	one-sided	synchronous	NST G2 DCIS G2 LCIS	Surgical Radiation Hormonal therapy	alive
2	multifocal	one-sided	synchronous	LELC G3 NST G3 DCIS G2	Surgical Radiation Hormonal therapy	alive

Lymphoepithelioma like – NST (ductal) G3 with pronounced stromal reaction (according to the current WHO classification)