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CONCORDANCE BETWEEN CLINICO-MORPHOPATHOLOGY AND IMMUNOHISTOCHEMICAL FEATURES WITH MOLECULAR SUBTYPES OF INVASIVE BREAST CARCINOMA

Abstract

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List of abbreviations

ABC - Complex Avidin-Biotin
aCGH - Comparative genomic hybridisation microarray
ADH - Smooth muscle actin.
ADH - atypical ductal hyperplasia
ADN - Acid Deoxiribonucleic
AJCC - American Joint Committe on Cancer
AR – Androgenic receptor
ARN – Ribonucleic acid
CDI – Invasive ductal carcinoma
CDIS – in situ ductal carcinoma
CEA –Carcino-embrionar antigen
c-erbB2 – Human epidermal growth factor 2
CISH – chromogenic in situ hybridization
CK – Citocheratin
CLI –Invasive lobular carcinoma
CLIS - in situ lobular carcinoma
BC - breast carcinoma
Cox2 – Ciclooxigenaza 2
EGFR – receptor of human epidermal growth factor 1
EMA – epithelial membranar antigen
ENS - Enolaza neuron specific
ER – Estrogen receptor
FISH – fluoren situ hybridization
GCDFP -15 – gross cystic disease fluid protein 15
HCL - Acid clorhidric
HE - Hematoxilin-eozin
HER 2neu – Human epidermal growth factor 2
IHC – immunohistochemical examination
LSAB - Method "Labed StrepAtvidin Biotin"
LumA – luminal A
LumB – luminal B
WHO – Whord human organisation
PAP - Peroxidaz-Antiperoxidaz
PAS- Acid Periodic Schiff
PR – progesteron receptor
SMA – Smooth muscle miosin
SMM-HC – Smooth muscle miosin – heavy chain
TNP – Triplenegativ
TTF1 – Thiroid transcription factor 1
VEGF – Endothelial growth factor
VG – van Gieson

KEY WORDS: invasive breast carcinoma, molecular subtypes, immunohistochemistry, chromogenic in situ hybridization.

INTRODUCTION

Worldwide, cancer remains a major health problem, and despite the huge efforts for development of screening methods for early detection and effective treatment of malignancies, the number of victims of this disease is growing. Thus, if in 2008 the incidence of cancer was 12.7 million, with a death rate of 7.6 million, according to data published by GLOBOCAN 2008 to 2012 found an incidence of 14.1 million, which 1.7 million is recorded with breast cancer (ranking second after lung cancer), and a mortality rate of 8.2 million, about half of these cases are found in economically less developed countries and the most common cause being lung, liver and stomach [1]. In this global context, Europe is also characterized by an increased incidence of cancer of the 3.2 million new cases per year in 2008 to 3.45 million in 2012 (except in cases of non-melanocytic skin cancer) by 53 % for males and 47% in women, and the death rate from 1.7 million in 2008 to 1.75 million in 2012 (56% for males and 44% for females) according to the International Agency the World Health Organization for Research on Cancer in Lyon, France [2,3]. Of all cancers, for females, the number of cases of breast cancer recorded in 2012 was the highest growth in the world, with 1.7 million new cases (20% more than in 2008) and 522 000 deaths (14% more than in 2008), the main cause of death in women [1]. In Romania, the reports made in 2012 places the female breast in a "forefront" position in 2012, as in most of Europe, so the incidence of new cases of breast cancer, with 89,800 cases and the number deaths being recorded 32.400, more than 2008 [2,4]. Increased efforts are needed for further research in malignant breast pathology, as in the other pathologies, aiming to reduce morbidity and mortality of breast cancer.

Histopathological classification of current publications disseminated by WHO in 2003 and then in 2012, includes 20 main types and 18 subtypes and is recognized and accepted worldwide as one of the most paramount prognostic factors along with other factors (histopathological grade staging pTNM, the maximum size of the tumor, limphonodular status, immunohistochemical expression of ER and PR, overexpression or amplification of the oncogene HER2neu) whose prognostic and / or therapeutic has been demonstrated by numerous research and clinical trials. However there are small drawbacks, since most cases of BC (approx. 75%) are included in the category of CDI-NST, histopathological class that although it has similar morphological features, however, are characterized by a high variability of biological behavior and therapeutic response [5]. This finding is demonstrated since 1998, when it was observed that only 30% of patients with breast carcinoma who were treated with chemotherapy or hormone therapy had a real benefit, the remaining 70% could survive without these treatments [6,7]. Also, other histopathology subtypes (CLI, CM mixed or other rare, included in "special subtypes"), are represented by a small percentage of cases and provide limited prognostic and predictive information, requiring further studies to elucidate the complex molecular mechanisms involved in carcinogenesis.

The development of molecular techniques and genetic performance of "high-throughput" in decades with the use of analytical methods for interpreting complex data allowed analysis of numerous genes extracted from a single sample and develop a new classification system for breast carcinoma based the transcriptome changes [8,9,10] and genomic (DNA copy number changes) [11, 12]. Such molecular classification of breast cancer originally proposed by Perou et al [8] and Sorlie et al [9] is constantly improving with the developmental means of investigation and numerous retrospective and prospective studies.

The need of knowing the mechanisms that lead to serious biological behavior of tumor lesions require extensive research, covering a large, representative samples of the population. But

this "boom" is hindered by the enormous costs of molecular cytogenetic techniques and the difficulty posed to be applied to a large number of patients has led to the definition of these intrinsic molecular subtypes based on immunohistochemical expression of four biomarkers. Thus St Gallen Consensus from 2011 and 2013 [13,14] stated their four molecular subtypes: luminal A, luminal B (with two subtypes: Luminal B - HER2positive and LuminalB - HER2negative), HER2positive and triple negative (TNP), each characterized by the expression of a unique set of genes with distinct predictive and prognostic implications. The first two are associated with a better prognosis and favorable response to hormonal therapy in contrast with tumors classified as HER2 positive and TNP subtypes, which are characterized by an aggressive biological behavior with lack of response to hormone therapy or chemotherapy. Viability of immunohistochemical examination as a "surrogate" method for molecular gene profile was proven by previous studies, this facilitating the clinico-morphological characterization of molecular subtypes for different samples of populations, sometimes yielding different results.

The current study is structured in two parts, divided into 10 chapters. The first part contains data from the literature on the embryology, anatomy, physiology, morphology of mammary gland and epidemiology, histopathological aspects of breast cancer. It also summarizes the latest information on molecular toxonomia CM. The second part, divided into five chapters, relates to our contributions in the study of clinico-morphological, immunohistochemical and molecular breast carcinomas.

PERSONAL CONTRIBUTIONS

Chapter VI - Motivation, scope and objectives of doctoral study

VI.1. The motivation for doctoral study

Morphological and biological BC is a heterogeneous disease that includes numerous subtypes [15]. WHO Classification of BC, known and accepted worldwide, is indispensable for correct histopathological diagnosis to trace the main lines of specific treatment. However, it was found that the BC classification system is not enough, there are many situations in which malignant tumor lesions which shows the same morphological feactures had a different biologic behavior [5]. It is these situations of discrepancy in terms of the ability to predict a favorable response in the treatment of similar tumor lesions, from the point of view of clinical presentation and morphological and immunohistochemical features, have led to the need for development of a new taxonomy of BC which has provide additional information that allows tailored therapeutic protocol [16, 17]. Thus, genetic and molecular research done for breast cancer in the past decade have led to the shaping of a new system of classification of BC that is based on the modality of gene expression and DNA copy number alteration. They complement histopathological diagnosis and identify patients with poor prognosis or may benefit targeted treatments. The four main molecular classes determined after St. Gallen Consortium from 2013 are the luminal A, luminal B (with two subtypes: luminal B and luminal-B-HERpozitiv HERnegativ), HER2 positive and TNP[14].

Although the amount of information coming from this research is invaluable, however, studies on large groups of patients who follow correlations with clinico-pathological aspects and other known and proven biomarkers with prognostic and predictive role are low across Europe, including Romania, especially for Dobrogea area, providing a strong argument in making this doctoral study.

VI. 2. The purpose and objectives of doctoral study

The purpose of the current study is to identify molecular subtypes of breast carcinomas using "surrogate" immunohistochemical criteria based on a primary pannel of four biomarkers (ER, PR, HER2neu, Ki67), with setting of correlations with main clinico-morphological and immunohistochemical factors and implications on short time overall survival rate.

Doctoral study objectives consist of:

- clinical and morphopathological analysis of cases included in the study
- the immunohistochemical expression analysis of biomarkers used to assess cases included in the study.
- including cases on molecular classes and assess their morpho-immunohistochemical characterization.
- statistical analysis of data obtained.

Capitolul VII MATERIAL AND METHOD

VII. 1.1. Description of the study design

Personal research were based on a retrospective analysis of breast carcinomas operated and diagnosed in surgery clinic of Emergency County Hospital "St. Andrei "Constanta, on a period between 01.01.2008 and 31.12.2013, and who benefited from complete histopathology evaluation in the clinical pathology service of same hospital. For this retrospective study it has been used both the main register and the computerized database of Emergency County Hospital "St. Andrei "Constanta, and were retained the main clinical data (age, gender, anatomic seat of the tumor, date of diagnosis), pathological aspects represented by the type of specimen tissue (breast tissue or lymph nodes), histopathologic diagnosis, grade and pTNM stage of BM, complications occurred during the follow-up of the patients.

From 927 cases hospitalized, diagnosed and surgically treated in the Emergency County Hospital "St. Andrei" Constanta in the six years under study (639 - female, 33 - male) it was selected a main population sample including 293 breast carcinomas, representing cases where the histopathological diagnosis was performed on breast tissue and/or lymph nodes, and if there were tumors with extemporaneous examination, confirmation by histopathology of the pieces included in paraffin was mandatory. All cases from this main group benefited immunohistochemical analysis using a main pannel of four biomarkers (ER, PR, HER2neu, Ki67), to which were added other biomarkers either with a complementary role in refining histopathological diagnosis or molecular subtypes (CK5 / 6, EGFR, E-cadherin, Sinaptofizină, ENS, chromogranin, SMA) and the prognostic and predictive role (p53).

From this main group was chosen a second sample consisting of 239 BC that belonged to females diagnosed in the period 01.01.2008 - 31.12.2012 and postoperative follow-up could be made for a minimum period of 15 months supervision. Analysis of these patients was performed in order to determine the impact of classification of mammary tumors in specific molecular subtypes on short-term overall survival.

VII. 1.2. The criteria for eligibility and exclusion

Eligibility criteria

1. The cases recorded in the Emergency County Hospital "St. Ap. Andrei" Constanta who

received surgical treatment.

2. Cases with confirmed histopathological diagnosis of invasive breast carcinoma.
3. The cases with complete immunohistochemical examination of four antibodies (ER, PR, HER2-neu, Ki67).

Exclusion criteria

1. Cases in which the histopatolgic diagnosis was represented by another type of malignancy of the breast.
2. Cases in which histopathological diagnosis and / or immunohistochemistry established the presence of breast carcinoma "in situ" with or without microinvasive carcinoma
3. Cases diagnosed with BC undergoing lumpectomy or tumorectomy or in another hospital and clinical-pathological data were not available or who received chemotherapy prior to radical mastectomy.
4. Cases with incomplete primary immunohistochemical pannel.
5. The cases confirmed with diagnosis of Paget's disease without an invasive breast carcinoma component.

VII.2. Histopathological material processing

The cases studied were evaluated both in terms of macroscopic, microscopic, immunohistochemical and by molecular cytogenetics. For this study were analyzed surgical specimens obtained by mastectomy, sectorectomy and tumorectomy. Surgical pieces were first subjected to macroscopic examination. Subsequently, the pieces were processed through successive stages until histopathological preparation stage. After fixation in 10% formalin solution for 24 hours, the tissue fragments were included in blocks and dehydrated in acetone solution for 24 hours. Sections with a thickness of between 4 microns were plated onto slides and for dewaxing have been introduced in methanol. The blades were stained using Hematoxylin-eosin staining and van Gieson. The procedure was completed by clarifying and installation of blades. After that the slides thus obtained were examined in a Nikon E600 microscope.

The immunohistochemical study was performed on paraffin blocks which were carried out the necessary processing sections of the classical histological hematoxylin-eosin. Through their cutting microtome at three levels, were obtained serial sections with a thickness of 4µ and were applied on slides treated with adhesive (polylysine). Further En Vision (Dako) immunohistochemical method was used as polymer amplification technique work, which is an indirect method of two-stroke and has been used as chromogenic Diaminobenzidine - DAB (DAKO).

The immunohistochemical reactions were completed by viewing brown investigated antigens (nuclear staining, cytoplasmic or membrane, depending on the antigen investigated). To achieve quality control was performed either positive and negative external control (normal or pathological sections) or internal positive control. (Table no. 1).

Tabel nr.1. Immunohistochemical biomarkers used

Primar antibody	Clona	Diution	Antigen retrieval	Positive control
Primary Biomarkers				
ER	Rabbit monoclonal	Ready to	EnVision ^{TM} FLEX Target	Breast cancer

Primar antibody	Clona	Diution	Antigen retrieval	Positive control
	antibody 1D5	use	Retrievel Solution, high PH	
PR	Mouse monoclonal antibody PgR 636	Ready to use	EnVision ™ FLEX Target Retrievel Solution, high PH	Breast cancer
HER2neu	Rabbit Imunoglobulin HercepTest	Ready to use	Epitope Retrieval Solution supplied by HercepTest™ kit	Breast cancer
Ki-67	Mouse monoclonal antibody MIB-1	Ready to use	EnVision ™ FLEX Target Retrievel Solution, high PH	Tounge
Secondary biomarkers important in diagnostic or with prognostic / predictive role				
P53	Mouse monoclonal antibody DO-7	Ready to use	EnVision ™ FLEX Target Retrievel Solution, high PH	Breast cancer
EGFR	Mouse monoclonal antibody EGFR.113	Ready to use	Proteinase K	Scuamous cervical cancer
Ck5/6	Mouse monoclonal antibody clona D5/16 B4	Ready to use	EnVision ™ FLEX Target Retrievel Solution, high PH	Tounge
Ecadherina	Mouse monoclonal antibody clona NCH 38	Ready to use	EnVision ™ FLEX Target Retrievel Solution, high PH	Normal breast tissue
Cromogranin A	polyclonal rabbit	Ready to use	DakoCytomation Target Retrievel Solution, high PH	Brain tissue
Synaptophysin	Mouse monoclonal antibody, clona SY38	Ready to use	DakoCytomation Target Retrievel Solution, high PH	Brain tissue
Neuron-specific enolase NSE	Mouse monoclonal antibody clona BBS/NC/VI-H14	Diluție 1:100	DakoCytomation Target Retrievel Solution, high PH	Brain tissue
SMA	Clona 1A4	Ready to use	EnVision ™ FLEX Target Retrievel Solution, high PH	Salivary gland

Vendor - Dako, Denmark

VII.4. Determining amplification of *HER2neu* gene by chromogenic hybridization in situ (CISH)

Cases where the evaluation by immunohistochemistry of HER2 neu oncoprotein were equivoc with a HercepTest score 2+, were further analyzed for gene amplification of *HER2neu* or *c-erbB-2*, a proto-oncogene located on chromosome 17 (17q11.2-21), especially through the technique of chromogenic in situ hybridization (CISH) and fluorescence (FISH), numerous studies demonstrating a high concordance between CISH and FISH, with values from 85% [18] to 100% [19, 20]. The gene detection HER2neu chromogenic in situ hybridization it's done by using the ZytoDot SPEC HER2 Probe Kit from ZytoVision.

The advantages of CISH to FISH:

1. possibility ordinary optical microscope examination
2. identification of gene amplification Her- 2 / neu in the context of morphology BC
3. Permanent signals that can be archived and reviewed whenever necessary
4. lower price for reagents
5. easier to assess as FISH

Table no. 2 Criteria for assessment and quantification of gene status HER2 /neu

Gene amplification HER2 / neu with high level	> 10 children or a large group of amplification per nucleus in more than 50% of cancer cells.

Gene amplification HER2 / neu with low level	5-10 children or a large group of amplification per nucleus in more than 50% of cancer cells
Absence of HER-2 /neu gene amplification	1–5 puncte ale genei *HER-2/neu* prezente per nucleu în >50% din celulele carcinomatoase în zona selectată

VII.5. Surrogate immunohistochemical criteria for defining the molecular subtypes

Intrinsic molecular subtypes	Molecular subtypes using immunohistochemical criteria "surrogate"	
Luminal A	**Luminal A –like**	ER şi PR* pozitive + HER2 negative + low index Ki67 **
Luminal B	**Luminal B-like (HER2 negativ)**	• ER pozitive + HER2 negative and at least one of the following criteria; high Ki67 index • low or negative for PR expression
	Luminal B-like (HER2 pozitiv)	ER pozitive Overexpression or amplification HER2neu Any Ki67 index or expression of PR
Overexpression of *cerb-B2*	**HER 2 pozitiv** (enrich, **non-luminal)**	Overexpression or amplification HER2neu ER and PR negative
Basal-like	**Triplu negativ (ductal)**	ER and PR negative; HER2 negative

∗ To define the luminal subtype A PR expression value must be greater than 20% [21].
∗∗ Cut-off value for Ki-67 is 14% [14, 20].

VII.6. Data processing and statistical analysis

Statistical analysis of clinical, histological, immunohistochemical and molecular techniques results was performed using SPSS statistical platform (Statistical Package for Social Sciences; eng - Statistical Package for the Social Sciences). Descriptive statistical analysis provided information on average and median for continuous variables and proportions or rates for qualitative variables. Establishing correlations between molecular subtype of BC and main clinico-pathological and immunohistochemical factors was performed using various statistical tests either parametric-type, when the dependent variables had a normal distribution or the non-parametric when normality condition was not satisfied. Thus, the following statistical methods were used: ANOVA (one way analysis of variance) for continuous variables (age, the maximum diameter of the tumor); Chi-square test analyzed correlations with categorical variables, Fisher's exact test was used when the conditions of the Chi square test were not met (over 20% of cells with value less than 5); Kruskal-Wallis test performed correlations with ordinal variables such as tumor stage, histopathological type. To estimate the correlations between two variables were used correlation coefficients: Pearson coefficient (r) and Spearman coefficient (r_s).

Short-term overall survival analysis was performed for a subgroup of patients enrolled in the study over a period of five years and the period of survival after the diagnosis was made during a period of at least 15 months. Survival curves were obtained using the Kaplan-Meier method, and to analyze the differences in survival between the molecular classes, from the point of view of statistical log-rank test was used. Multivariate analysis by Cox model proportional hazards regression was used in order to determine associations between survival time and the independent variables (age, sex, histopathological subtype, grade) and to obtain 95% confidence interval.

Statistical analysis was performed using the non-directional hypotheses (two-tails) and statistical significance was considered to be worth less than 0.05; values between 0.05 and 0.07

were considered statistically significant intermediate value, and values of p = 0.00 were reported as p <0.01 [40].

VIII.1. Results and discussion based on clinico-morphological study

In the period 01.01.2008 - 31.12.2013, the Service Clinical Pathology Clinical Emergency Hospital "St. Andrei "Constance were diagnosed by pathological examination complete a number of 927 benign and malignant tumors of breast, involving both sexes. Mammary carcinomas (in situ and invasive) predominated, with a frequency of 58% (Fig. no. 1), but with a disproportionate sex ratio, female "dominating" the spectrum of malignant lesions of the breast with a percentage of 95%.

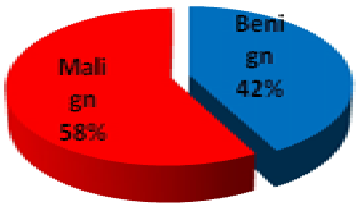

Fig. no. 1 The proportion of malignant and benign tumors of the mammary gland during the study

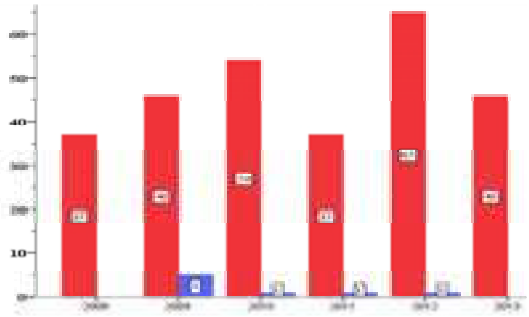

Fig. no. 2 The number of cases by year of diagnosis and sex -woman / male

Of these, based on the eligibility criteria in the present study were included 293 cases of which 285 cases (97.3%) belong to the female sex, the rest being observed in males (8 cases). Analysis of cases by year of inclusion in the study, and gender (Fig. no. 2) of the main batch of patients, reveals a peak of cases for females in 2012, with a frequency of 22.8%, followed by the recorded in 2010 with 18.9% of cases. The average age of patients in the study group is 60.55 ± 11.4 years (28-87), the majority (80%) having a postmenopausal hormonal status and recorded the average age for males is 63.8 ± 1.2 years (58 -78), higher than that observed in women. The difference between the mean diameter of tumor lesions belonging to the female sex 2,69 ± 1.9 cm (1 cm - 14 cm) did not differ from that obtained for the cases belonging to males 2.71 ± 1,2 cm (2-5cm).

In terms of histopathological classification it was noted a wide range, for both sexes, the CDI-NST having a frequency of 67.0% of cases for feminine gender and 87.5% of those seen in males. With a rate of 7.2% (19/293) were identified mixed carcinoma cases, mostly composed of CDI-NST and CLI. Special pathological forms were observed with a much lower than CDI-NST and these are, according to WHO classification 2012: CLI - 19 cases; mucinous carcinoma and invasive cribriform carcinoma each with 10 cases; medullary carcinoma was recorded in 6 cases; metaplastic carcinoma in 7 cases; adenoid cystic carcinoma and tubular carcinoma each with 3 cases; carcinoma with features neuroendocrine differentiation -2 cases; apocrine carcinoma, clear cell carcinoma and secretory carcinoma each with one 1 case. Association with carcinoma in situ component was observed in the amount of 57.1% morphologically often found in the literature, with 80% of cases reported to [22,23].

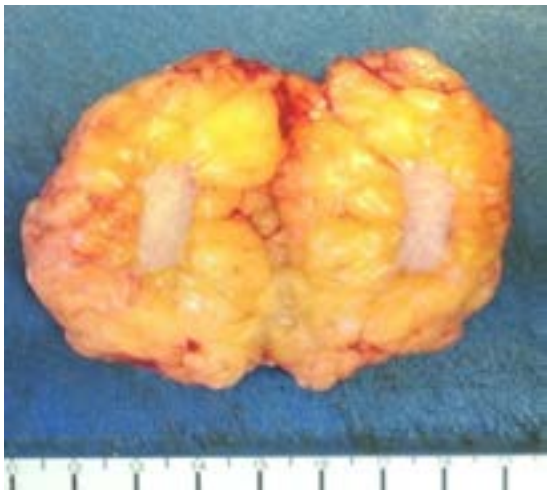

Fig. no. 3 CDI- NST – Macroscopic appearance - nodular lesion with stellate shape, high consistency (case no. 144, Original)

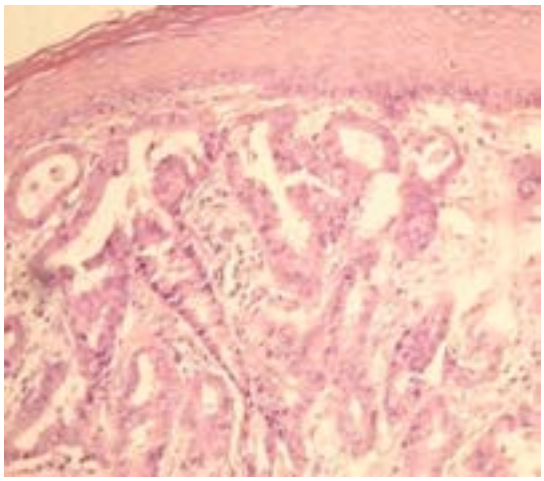

Fig. no. 4 CDI NST well differentiated G1 morphological aspect (HE 4x, Case no. 262, original)

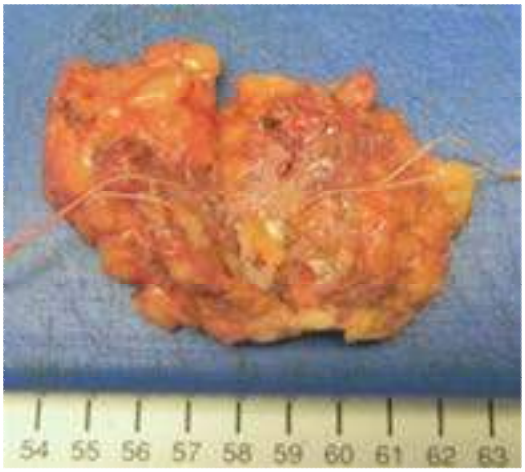

Fig. no. 5 CLI - Imprecisely defined tumor lesion, with infiltative peripheral edges, whitish-gray color (Case No. 226, original)

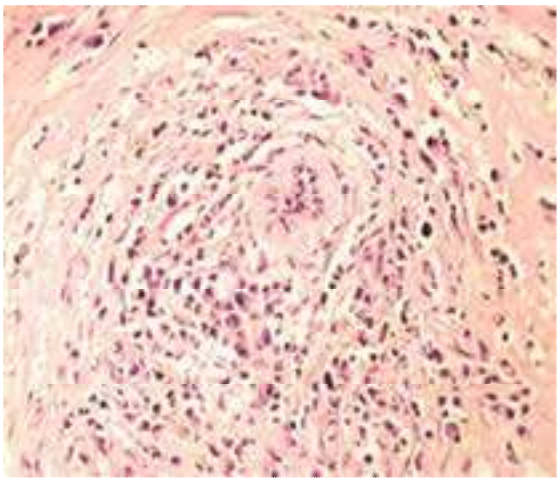

Fig. no. 5 Classic CLI - discoezive tumor cells with "targetoid" arrangement around a normal duct (HE 4x, Case no. 226, original)

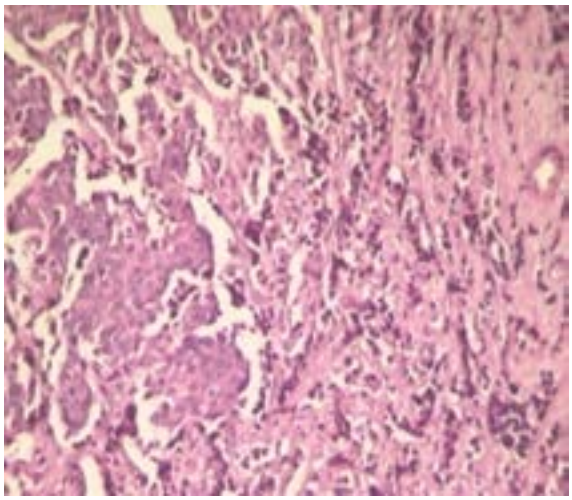

Fig. no. 6 Mixed BC - malignant epithelial proliferation with mixed pattern: CDI -NST and CLI (HE 10x, Case 131, original)

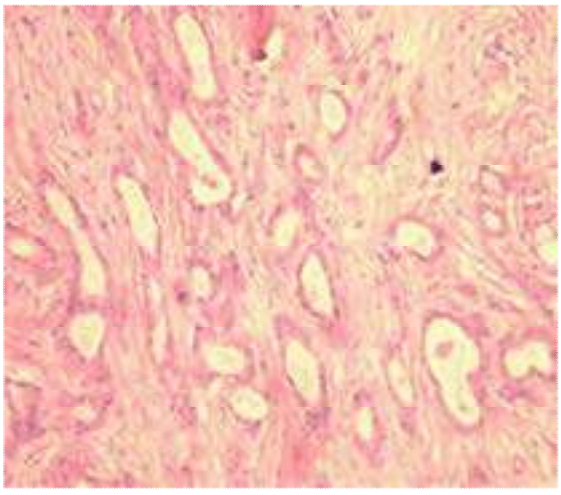

Fig. no. 7 Invasive tubular carcinoma - tubular structures, round or oval, angulated shape, arranged in a dezmoplazic stroma (HE 4x, case no. 81, original)

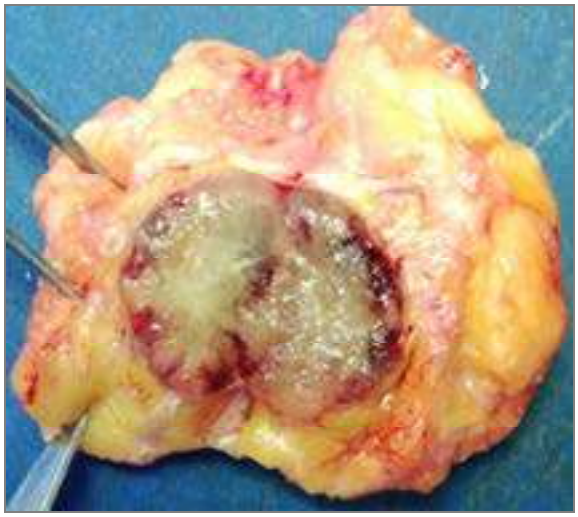

Fig. no. 8 Mucinos- carcinoma – macroscopic appearance: translucent, whitish gray tumor lesion with medium consistency (Case no. 138, original)

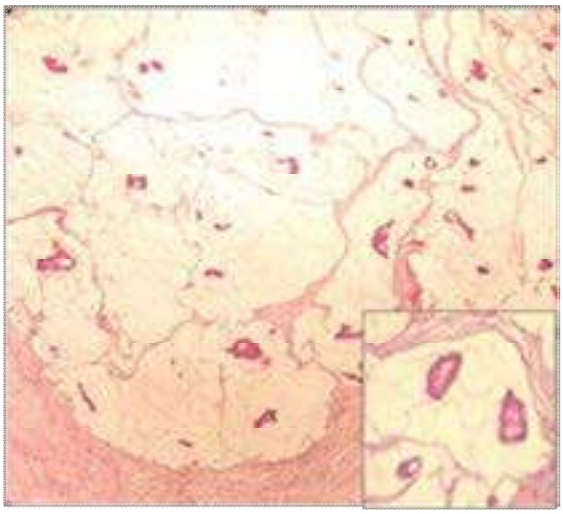

Fig. no. 9 Pure mucinous BC - type A hypocelular (HE, 4x; in box HE 10x, case no. 138, original)

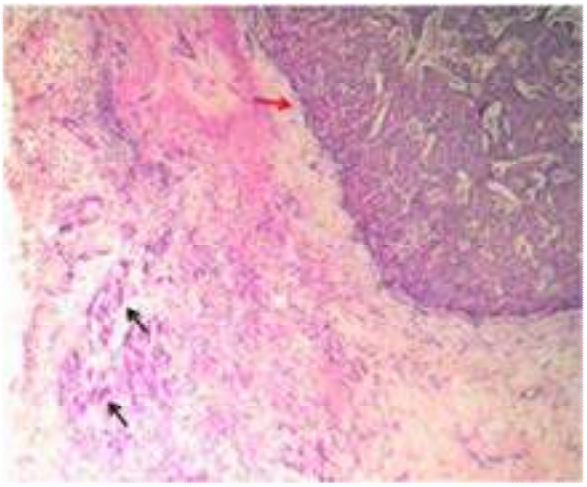

Fig. no. 10 Intraductal papillary carcinoma of solid type (→) associated with CDI-NST, G3 (→) (HE 4x, case no. 283, original)

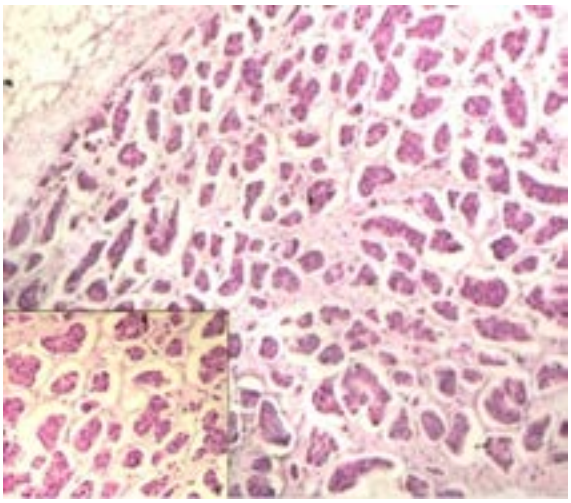

Fig. no. 11 Micropapillary invasive carcinoma - proliferation of tumor cells arranged in nests (HE 4x; in box HE10x, case no. 293, original)

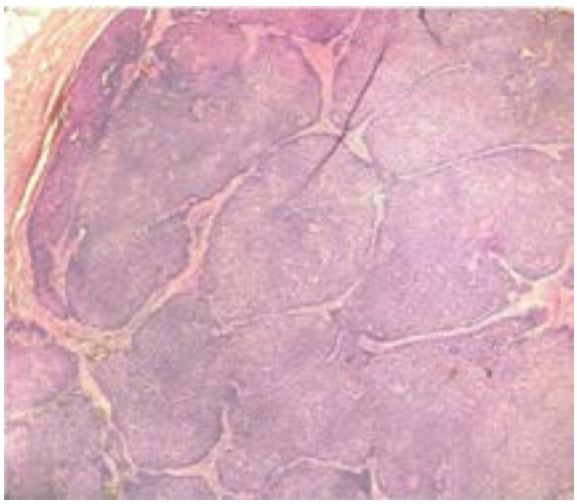

Fig. no. 12 Well differentiated neuroendocrine carcinoma, with solid pattern - proliferation of tumor cells arranged in solid sheets (HE, 4x original case, no. 88)

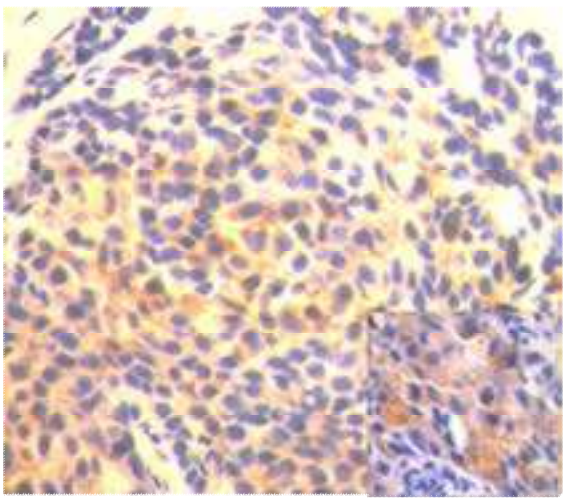

Fig. no. 13 Positive cytoplasmic immunostain for synaptophisin in more than 50% of tumor cells (IHC 10x, in box IHC 20x, case no. 88, original)

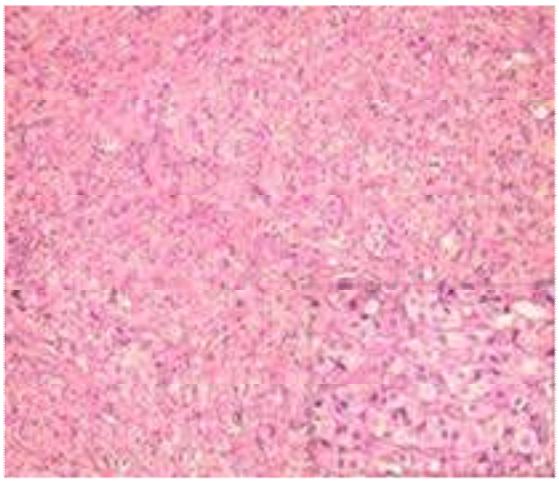

Fig. no. 14 Infiltrating apocrine carcinoma - malignant epithelial cell proliferation with abundant, eosinophilic cytoplasm (4x HE, HE 20x in box, case no. 177, original)

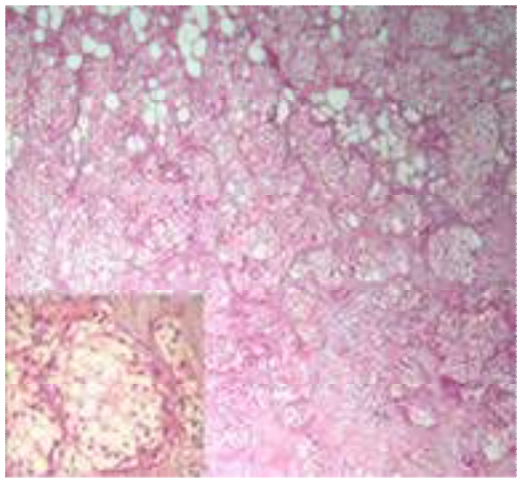

Fig. no. 15 Clear cell carcinoma - proliferation of glycogen-rich clear epithelial tumor cells arrange in small nests(HE 4x, in box HE 20x, case no. 236 original)

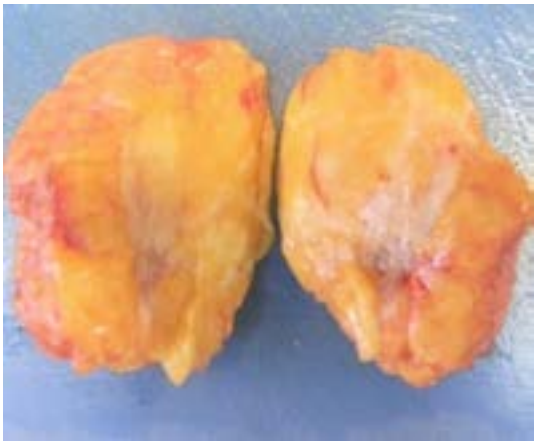

Fig. no. 16 Adenoid cystic carcinoma, - macroscopic appearance (case. No. 136, original)

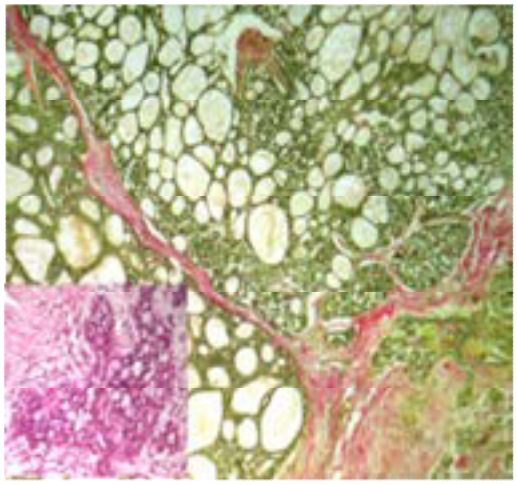

Fig. no. 17 Adenoid cystic carcinoma (VG 4x, in box 10x HE, case no. 288, original)

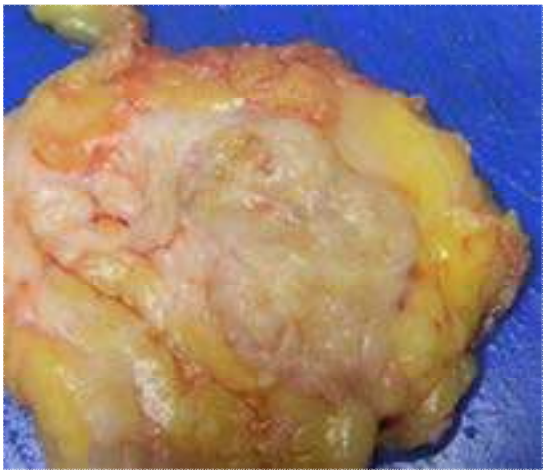

Fig. no. 18 Medullary carcinoma – macroscopic appearance- nodular lesion, imprecisely defined with solid, whitish-gray colour (case no. 178, original)

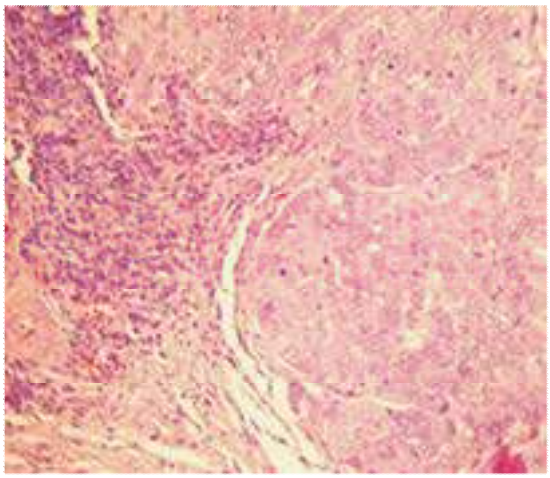

Fig. no. 19 Medullary carcinoma - invasively malignant tumor cells with syncytial pattern, surrounded by an abundand nflammatory infiltrate (HE, 4x case no. 178, original)

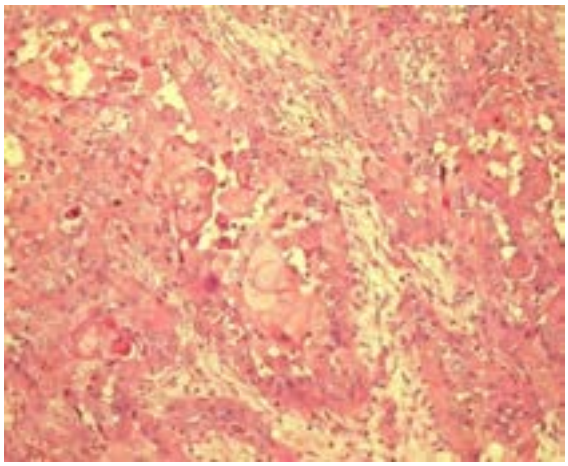

Fig. no. 20 Metaplastic carcinoma-squamous type -(HE 10x, case no. 237, original)

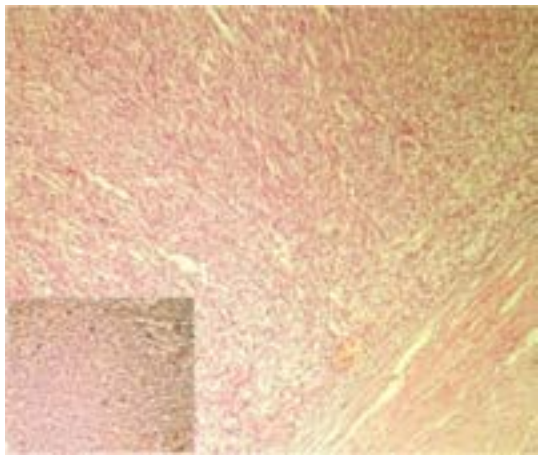

Fig. no. 21 Metaplastic carcinoma with fusiform cell (HE 4x), whose epithelial nature is demonstrated by a positive immunostain epithelial for CK5 / 6 (in box 10x IHC) (nr.287 case, original)

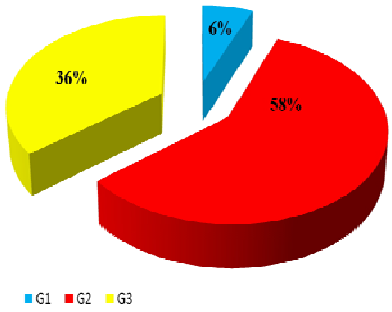

Fig. no. 22 Distribution of cases with morphological diagnosis of CDI-NST according to histopathological grade

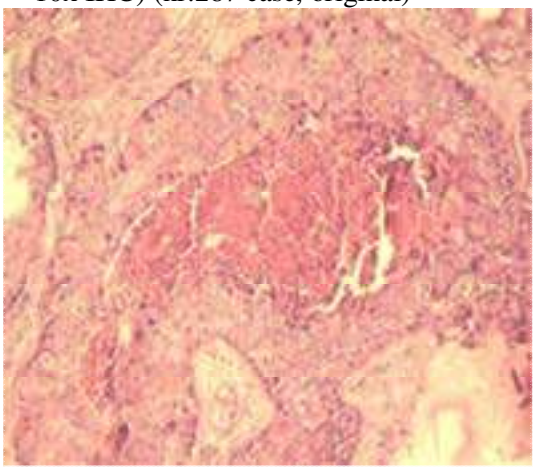

Fig. no. 23 Ductal carcinoma in situ - comedocarcinom - (HE 4x, Case No. 163, Original)

Peritumoral limfovascular invasion (Fig. nr.24), which is also an independent prognostic factor as well as the histopathological level, was observed in the current study in the amount of 30.0% of cases. Perineural invasion (Fig. nr.25) was found in 17.7% of cases, but not identify any statistically significant association with histopathological grade or tumor size. However there was a significant association with lymphovascular invasion, with a level of positive correlation (Chi square test, $\chi 2$ (2) = 21.7; p <0.0001; Spearman r = -0.347, p <0.0001).

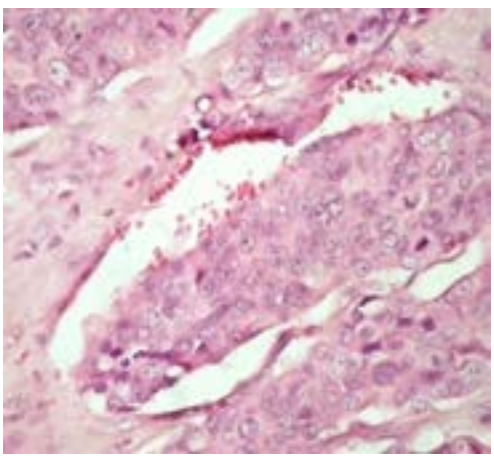

Fig. no.24. CDI-NST, G3. Limphovascular invasion (HE 20x, Caz. no.154, original)

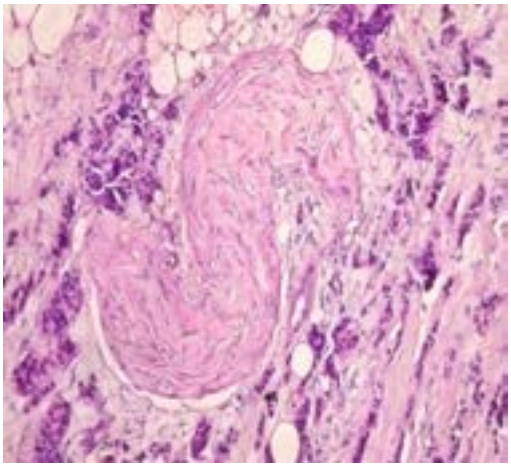

Fig. nr. 25 CDI-NST, G3. Perineural invasion of tumor cells (HE 20x, Caz. nr.170, Original)

VIII.2. Results and discussion based on immunohistochemical and molecular study

VIII.2.1. Immunohistochemical assessment of hormone receptors (ER and PR)

In the present study a negative expression was observed in 21.8% of cases for ER and 24.9% for PR, pointing out a positive imunomarcaj ER for all cases of male and 77.5% for females, higher levels than that for PR. For our study cases where the nuclear imunomarcaj positive for ER 84.8% of the cases observed a high expression of ER, more than 10% (Fig. nr. 26), the rest being represented by the absence of a nuclear imunomarcaj (Fig. nr. 27). **Expresion of PR** usually indicates proper functioning of signaling pathways activated ER-α and progesterone hormone is involved in regulating ER as certain cellular functions, including proliferation [24].

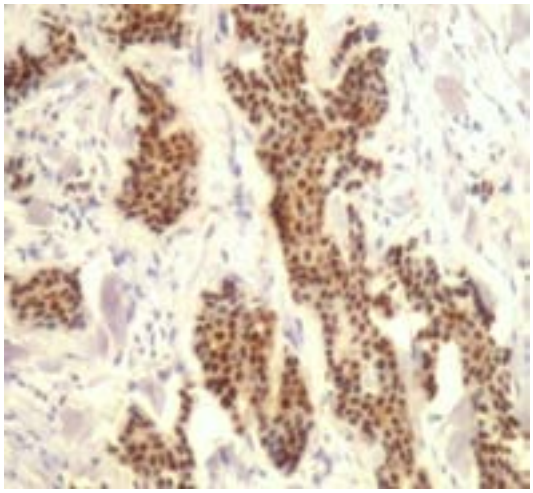

Fig. no. 26 Immunohistochemical exam – strong positive nuclear immunostain for ER (IHC 10x, caz no. 98, original)

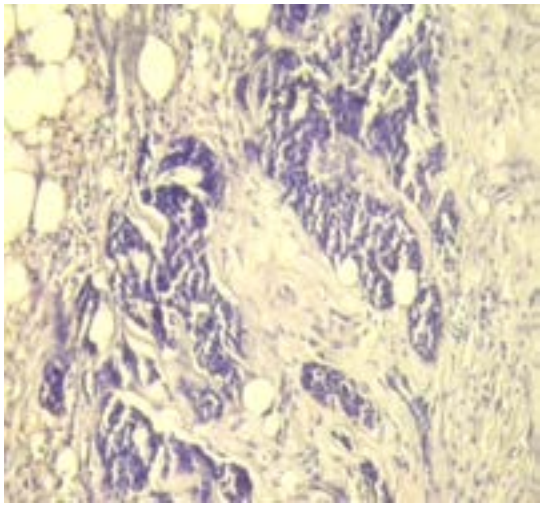

Fig. nr.27 Immunohistochemical exam – negative nuclear immunostain for PR (IHC 4x, caz nr. 221, original)

VIII. 2.2. The assessment of the immunohistochemical expression of HER2neu oncoprotein and / or the gene amplification of HER-2 / neu by chromogenic hybridization in situ

In the present study, immunohistochemical quantification of oncoprotein HER2/neu has been made for all the cases, of which 20.8% (61/293) overexpression has been observed to yield a positive score for IHC 3+ (Fig. nr. 28), and for 63.1% (185/293) of the cases was observed in the absence of overexpression give a imunomarcaj the 1+ and 0 (Fig. nr.29, 30). For 16.0% (47/293) of the cases it was observed a clear Score 2+ (Fig. nr. 31), this result required additional quantification of gene amplification of HER-2 / neu by CISH.

Thus of the 47 cases was on which it was made the determination of *HER2/neu* gene amplification, in 42,6% was a normal expression of HER2 / neu (Fig. nr.32) and in 57.4% (27/47) of its amplification was observed (Fig. nr. 33).

Following evaluation of amplification HER2/neu gene immunohistochemical results for cases with equivocal positive HER2neu status was observed in 30% of cases.

Distribution of cases according to histopathological diagnosis, highlights high frequency of cases with histopathological diagnosis of CDI-NST (85.2%), of which two cases were characterised by association between Paget's disease and CDI-NST, being followed, at a high distance, by mixed carcinomas in 5.7% of cases.

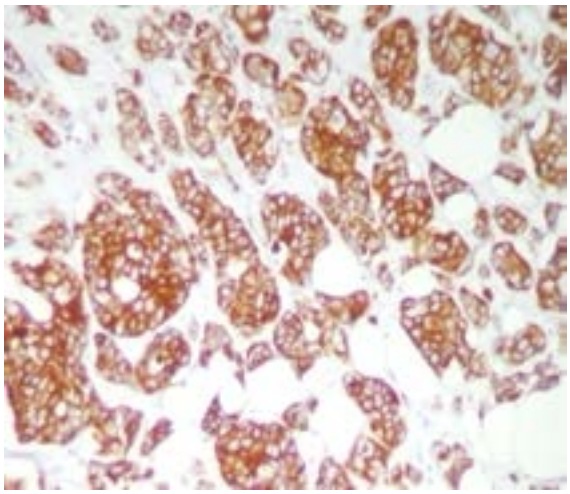

Fig. no. 28 Immunohistochemical exam for HER2 / neu - IHC 3+ score - imunomarcaj intense and continuous membrane in more than 30% of tumor cells (IHC 10x nr.281 case, original)

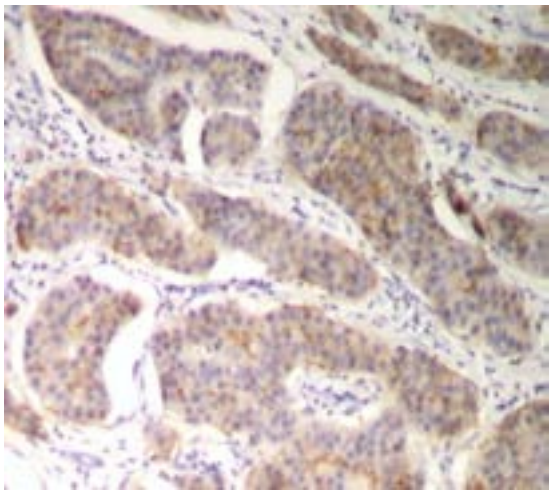

Fig. no. 29 Immunohistochemical exam for HER2 / neu – IHC score 1+ (negative) - imunomarcaj weak membrane less than 10% of tumor cells (IHC 4x, case no. 210, original)

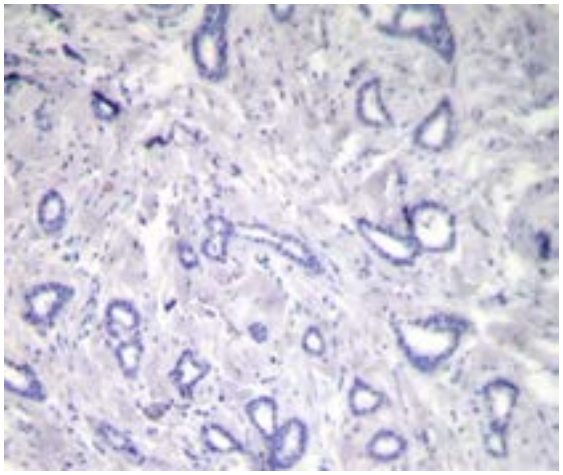

Fig.no.30 Immunohistochemical exam for HER2 / neu IHC score 0 (negative) membrane imunomarcajului -absenţa the tumor cells (IHC 4x, case no. 193, original)

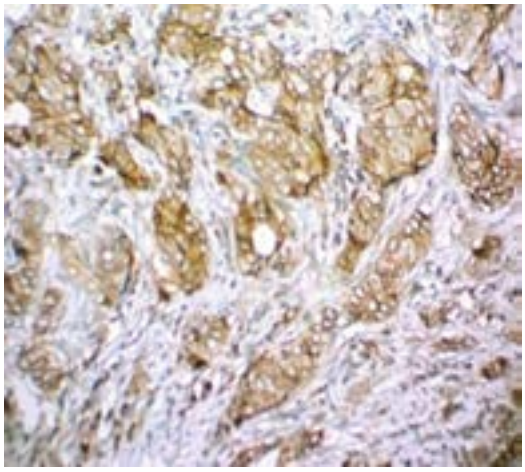

Fig. no. 31 Immunohistochemical exam for HER2 / neu IHC score 2+ (equivocal) - imunomarcaj membrane weak / moderate >10% of tumor cells (IHC 10x case no.198, original)

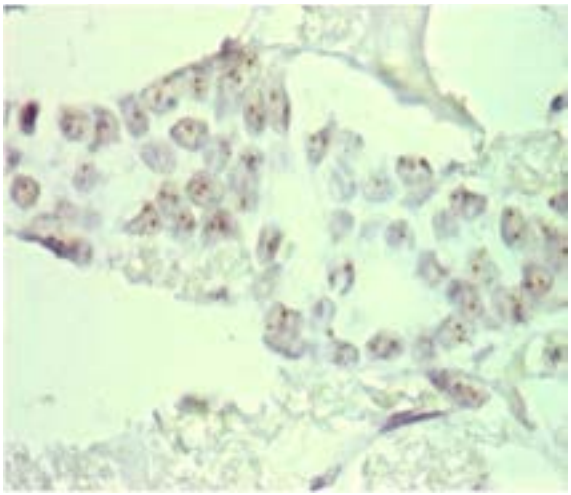

Fig. no.32 CISH - normal expression of HER2 / neu (Ob 40x, Case no. 245, original)

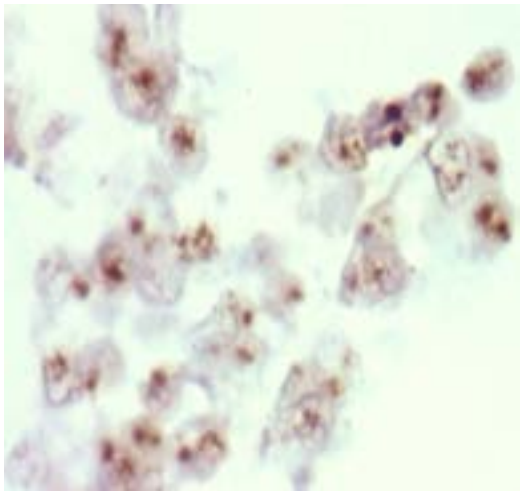

Fig. no.33 CISH - high amplification of HER2 / neu (Ob 40x, case no. 202, original)

VIII.2.3. Immunohistochemical evaluation of the biomarker Ki67 is carried out in all cases included in the study, yielding a negative index of 10.6% (31/293) of the cases and an increased to 34.1% (100/293) of cases. The remaining 162 cases (55.3%) was characterized by a decreased nuclear proliferation index (<20%), these cases are prevalent.

VIII.2.4. Immunohistochemical evaluation of p53 oncoprotein brings further information on the molecular characteristics of a tumor and the existence of a link directly proportional to predict prognosis and treatment. In the current study imunomarcajul p53 was evaluated in 222 cases, of which 33.1% (97/222) showed a positive reaction.

VIII.2.5. Immunohistochemical evaluation of biomarkers of basal type (EGFR and CK5 / 6)

In the current **study immunohistochemical expression of EGFR** was evaluated for 133 cases in which a positive response was noted for 39.1% of cases. This is higher than the results obtained by Tsutui et al and Walker et al in their studies that have shown expression of EGFR frequency of 36%, but in the literature are listed and lower rates of up to 7% of cases BC [19, 25]. The study by Lv et al demonstrated a positive correlation between overexpression of EGFR protein and EGFR gene amplification is associated with a poor prognosis [27].

Immunohistochemical expression of CK5/6 was analyzed in 45.4% of cases, a positive imunomarcaj (Fig. No. 92) was identified for 39.1% of them, a value that overlaps the data reported by similar studies which indicate a frequency of cases with positive immunostain for CK5/6 varying from 22% to 51% [28, 29].

Capitolul IX. Results and discussion on the molecular subtypes

IX.1. Molecular classification based on "surrogate" immunohistochemical criteria

Analysis of cases by the main biomarkers (ER, PR, Ki67, HER2neu), in conjunction with the application of the latest classification of molecular subtypes proposed by St Gallen Consortium (2013) [14], has highlighted an increased frequency of cases with luminal immunophenotype (luminal A and luminal B - 78.5% of cases) compared with the basal subtype and triple negative (non-luminal) represented 21.5% of cases. Of these cases, male was represented by only 8 cases, all of them belonging to luminal subtypes. For feminine gender the highest frequency of cases was recorded in luminal molecular subtype.

IX.2. Evaluation of breast carcinoma cases by luminal A molecular subtypes

In the main popuation sample, luminal A molecular subtype accounted the most frecquent subtype, 97.8% females with a mean age of 61.5 ± 11.6 years, and three cases of male gender with mean age of 58.3 ± 0.58. Similar studies have recorded different values for the average age of the molecular subtype luminal A 47.4% [30] to 61.4 years [31] for feminine, the result of our study fits in this variation. In terms of the distribution of cases by age groups was highlighted, for both sexes, an increased frequency of cases included in the age group 51- 60 years, with a percentage of 32.6% more compared to the distribution cases in the same category according to Spitale et al, who obtained a rate of 19.1% [32].

Morphological study of the luminal A subgroup of cases highlighted, in terms of histopathological subtype of WHO classification, CDI - NST as the most frequent diagnosis with 84 cases, followed by cases with special morphological subtypes: CLI - 13 cases and mixed carcinoma with 10 cases (Fig. nr.34-42). With a lower frequency it was recorded rare special histopathological forms: invasive tubular carcinoma (2.2%), invasive cribriform carcinoma (6.7%), pure mucinous carcinoma (6.7%), intraductal papillary carcinoma with component CDI invasive NST (0.7%) and neuroendocrine carcinoma (1.5%). Among them, the cases which belong to the

male gender are represented in terms of pathological diagnosis, the two cases of a case CDI-NST invasive cribriform carcinoma.

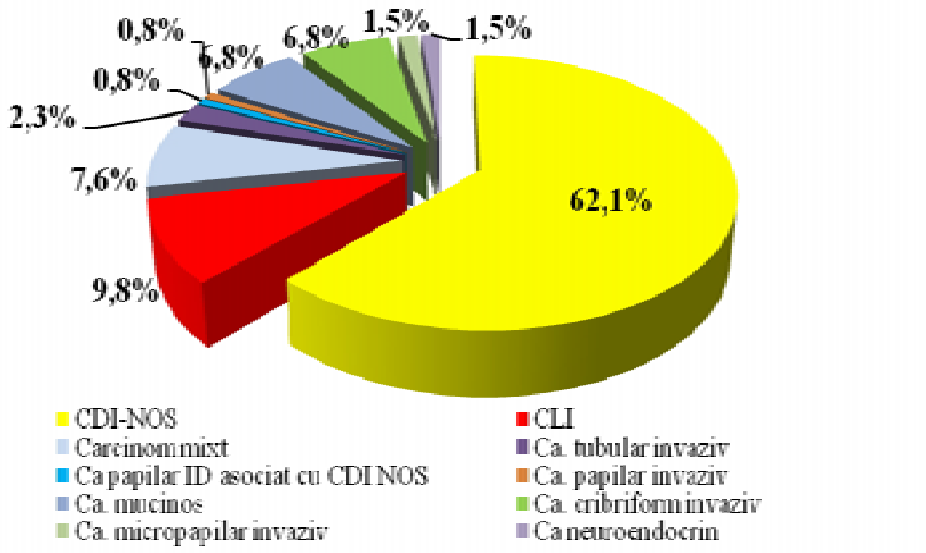

Fig. nr. 34 LumA distribution for female subtypes based on histopathological form of BM

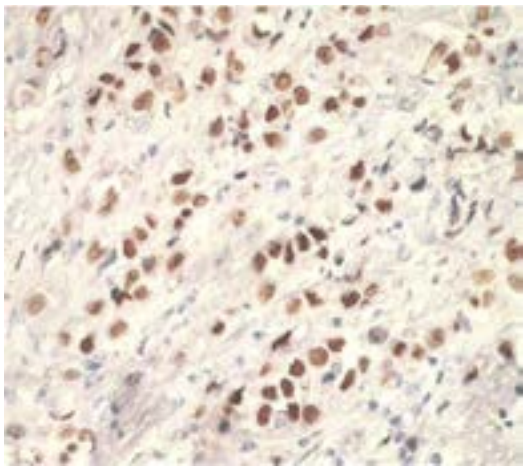

Fig. no. 35 Molecular subtype luminal A - pleomorphic CLI - strongly positive immunostain for ER (IHC 20x, case no. 75, original.)

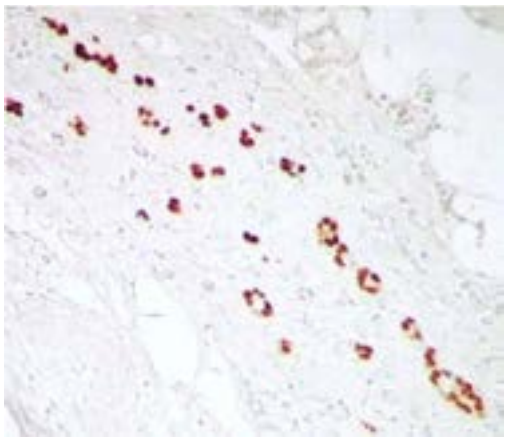

Fig. no.36 Molecular subtype luminal A - CLI tubulo-lobular subtype: strongly positive immunostain for PR. (IHC 10x, case no. 278, original)

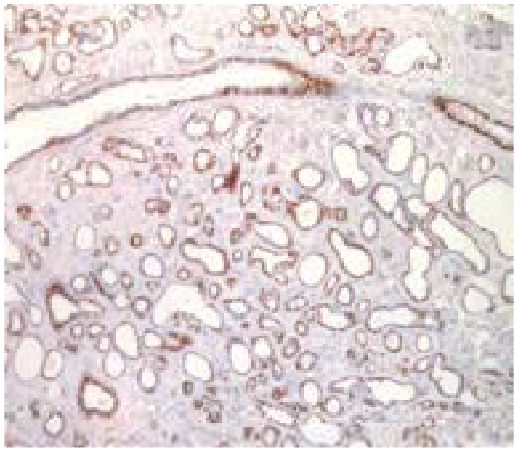

Fig. no.37 Molecular luminal A subtype - Infiltrative tubular carcinoma - 90%positive nuclear immunostain for RE (IHC 4x, case 174, original).

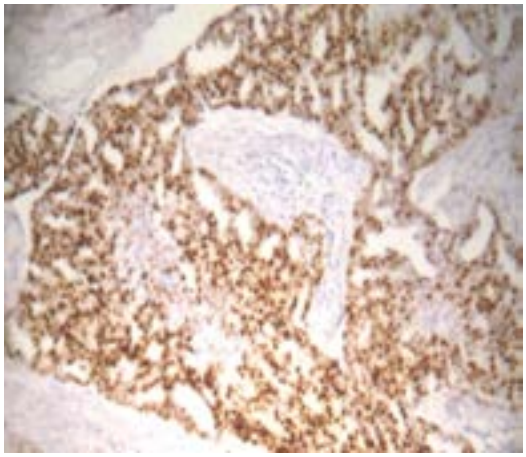

Fig. no.38 Molecular subtype luminal A invasive cribriform -carcinom: 90%positive nuclear immunostain for PR (IHC 10x case no.140, original)

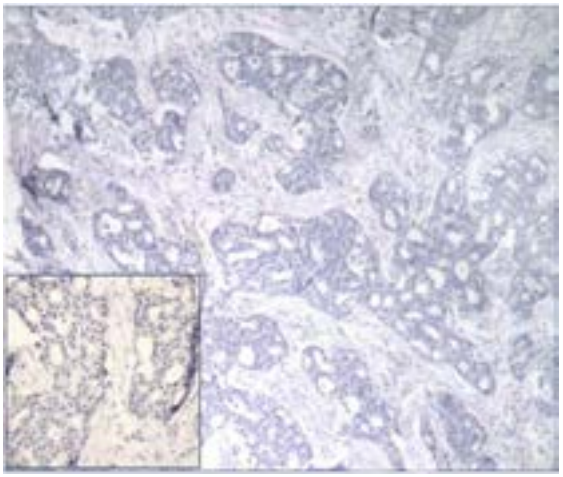

Fig. no.39 Molecular subtype luminal A - invasive cribriform carcinoma: negative immunostain for HER2neu (IHC 4x); box - no nuclear immunostain for Ki67 (IHC4x;in box 10x, case no. 87, original)

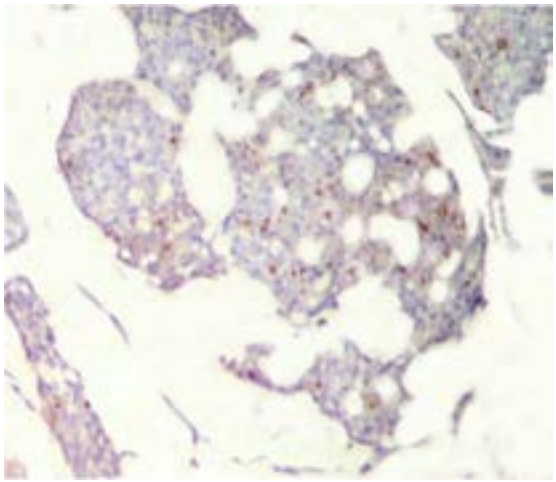

Fig. no. 40 Molecular subtype luminal A - pure mucinous carcinoma: low level for Ki67 index (IHC 10x; case no. 85, original)

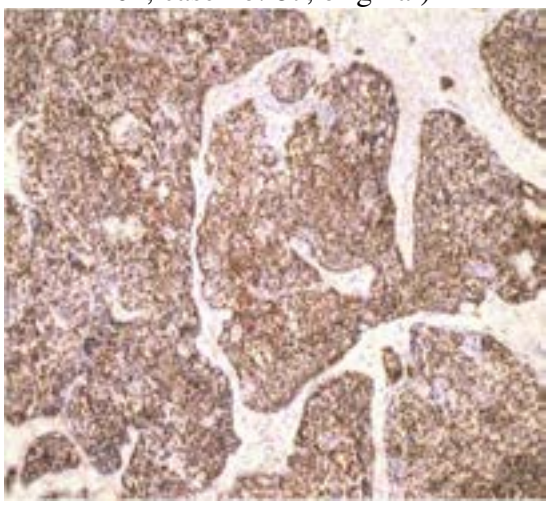

Fig. nr. 41 Luminal subtype A - solid neuroendocrine carcinoma. Intense and diffuse positive nuclear immunostain for ER (IHC 4x, case no. 88, original)

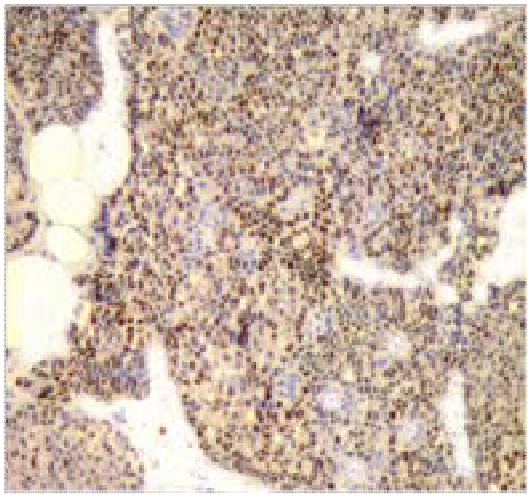

Fig. nr. 42 Luminal subtype A - solid neuroendocrine carcinoma. . Intense and diffuse positive nuclear immunostain for PR (IHC 4x, case no. 88, original)

Evaluation of immunomarker for p53 oncoprotein showed an increased frequency of positive cases for BC moderately or poorly differentiated (52.9% versus 41.2%) without evidence of a statistically significant association. Also was noted no statistically significant association between nodular status and immunohistochemical expression of p53 oncoprotein (Chi square test $\chi 2\ (1) = 1.491$; $p = 0.297$).

IX. 3. Evaluation of luminal B molecular subtype cases of invasive breast carcinoma

Breast carcinoma cases classified as luminal B subtypes based on "surrogate" immunohistochemical expression markers represent a rate of 32.4% (95 cases), ranking the second place in the molecular classification. This result is consistent with those reported in the literature although the frequency of this molecular subtype varies a lot from 6,9% [33] to 34 % [30]. In the study of Engstrøm et al on a 909g cases of BC, the recorded prevalence of luminal B cases - HER2negativ was 27.4% and that for luminal B-HER2 positive was 7.7%, similar to another study of Xue et al that, on a sample of 5809 patients, has achieved a rate of 30.4% for the first subgroup and 13.1% for the second [34, 35]. These results differ from those obtained in the current study in

which there is a reversal of the relationship between the two molecular subtypes of Luminal B, with a 11.9% prevalence of luminal B-HER2 negative cases and 20, 9% for luminal B-HER2positive cases which indicates the great heterogeneity of the molecular subtype.

Morphological assessment of BC cases of luminal B subtype showed an increased frequency of cases of CDI-NST (75.8%), the following places being mixed invasive carcinoma (8.4%), followed by the CLI and indraductal papillary carcinoma associated with an invasive component of CDI-NST, each with a frequency of 6.3%. The difference was represented by one case of pure mucinous carcinoma (1.1%) and two cases of micropapillary invasive carcinoma (2,1%) (Figura nr. 43-46).

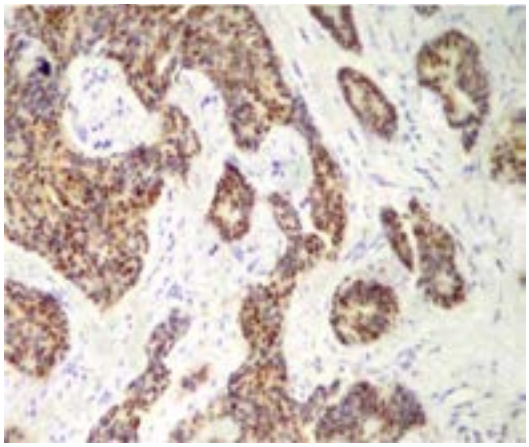

Fig. nr.43 Luminal B molecular subtype-HER2positive - CDI-NST, G2: intense positive nuclear immunostain for ER (IHC 10x case no.268, original)

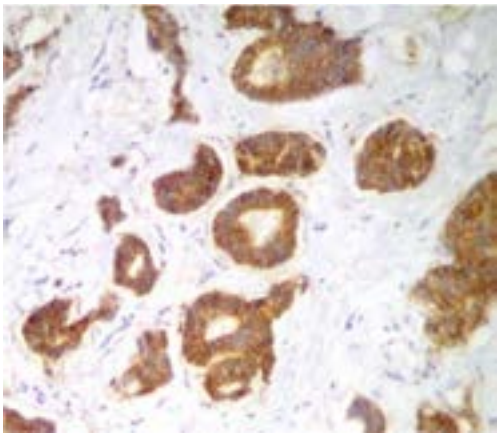

Fig. nr.44 Luminal B molecular subtype - HER2positive - CDI-NST, G2: strongly membrane positive immunostain for HER2neu (score 3+) (IHC 10x case no.268, original)

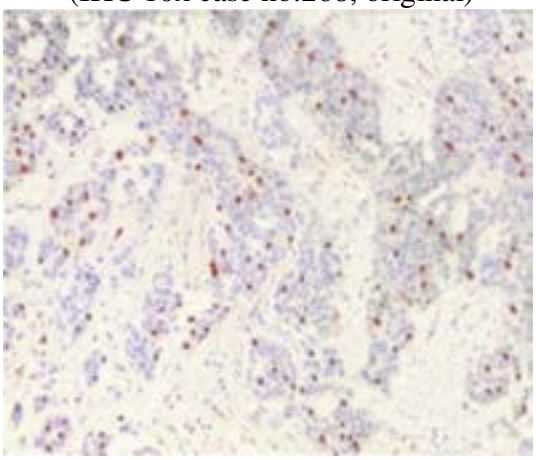

Fig. nr.45 Luminal B molecular subtype-HER2positive - CDI-NST, G2: positive nuclear imunostain for Ki67 (high Ki67 Index). (IHC 10x case no.268, original)

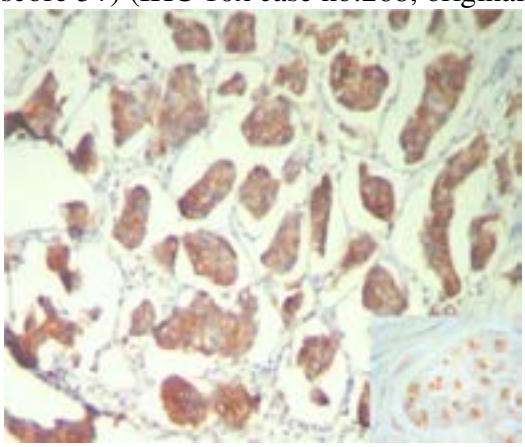

Fig. nr. 46 Luminal B molecular subtype - micropapilary invasive carcinoma: moderately positive and discontinuous membrane imunostain for HER2neu; in box - high amplification of oncogene *HER2neu* (IHC 10x; in box CISH 40x, case no. 247, original)

IX. 4. Evaluation cases classified as HER2-positive molecular subtype (non-luminal)

For the main group of patients, HER2-positive molecular subtype occupies the last place in terms of frequency with a 9.6% frequency and 28 cases as absolute number. In this subgroup all cases belong to the female gender with a mean age of 58.71 ± 10.5 years, most patients were included in the category 61-70 years (46.4%). Through morphological analysis of the cases assigned to molecular subtype was noted an increased frequency of 78.6% of cases with CDI-NST

histopathological form (22/28) followed by the association between Paget's disease and CDI-NST accounting 3 cases. Mixed carcinoma, clear cell carcinoma glycogen rich and infiltrating apocrine carcinoma were each represented by one case. (Fig. nr. 47-50)

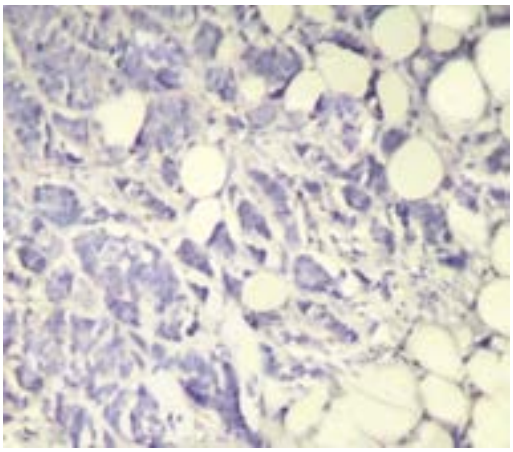

Fig. nr. 47 HER2 positive molecular subtype - poorly differentiated CDI-NST. Negative immunostain for ER. (IHC 4x case. no. 289, original)

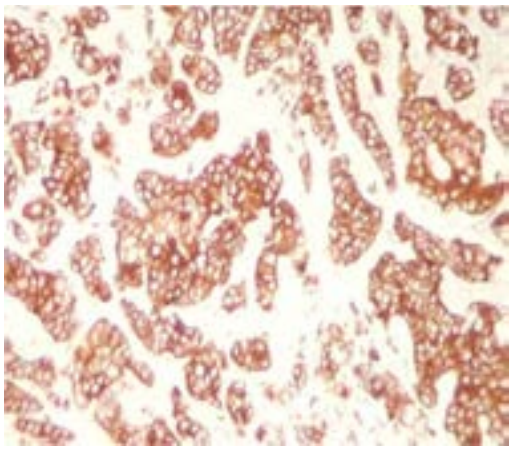

Fig. nr. 48 HERpositive molecular subtype - poorly differentiated CDI-NST - strongly positive immunostain for HER2neu - score 3+ (IHC 10x case. no. 289, original)

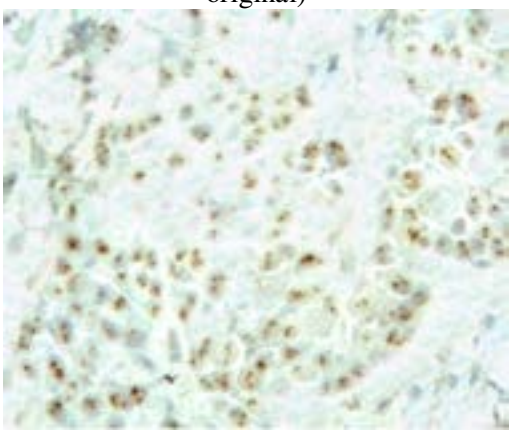

Fig. nr. 49 HERpozitive molecular subtype - moderate differentiated CDI-NST, High amplification of *HER2 / neu*. (CISH, 20x, case no. 213, original)

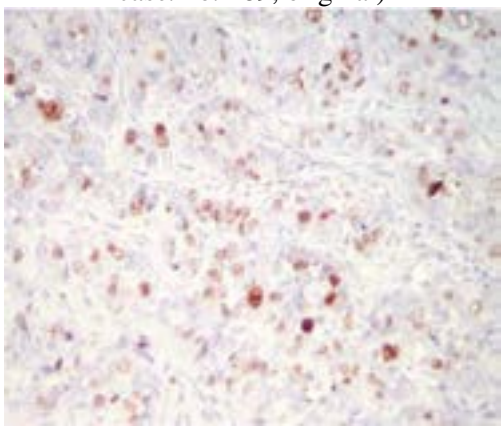

Fig. nr. 50 HERpositive molecular subtype - poorly differentiated CDI-NST - strongly positive immunostain for KI67 (IHC 4x, caz no. 265, original)

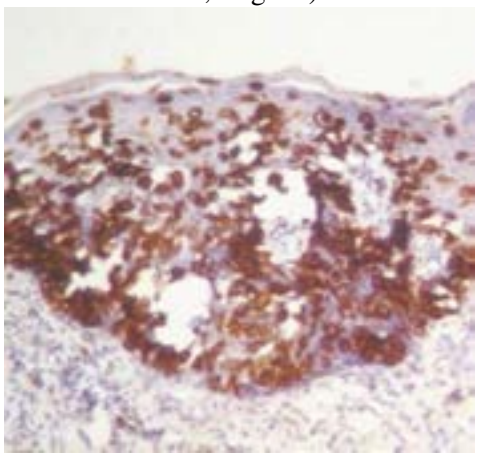

Fig. nr. 51 HERpositive molecular subtype – Paget's disease – strongly positive immunostain for HER2neu in the tumor cells (IHC 10x, case no. 222, original)

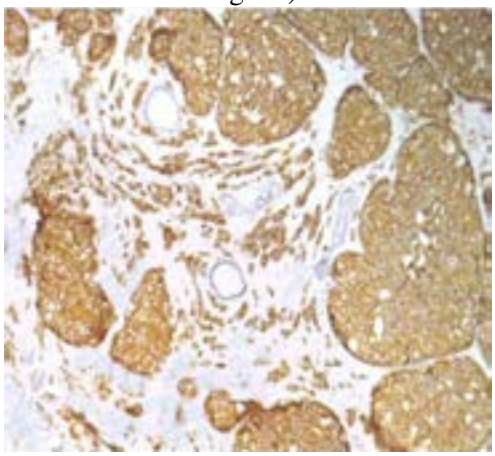

Fig. nr. 52 HERpositive molecular subtype – Paget disease associated with CDI-NST - strongly positive immunostain for HER2 (score 3+) both in the in situ component and the invasive component (IHC 4x, case no. 222, original)

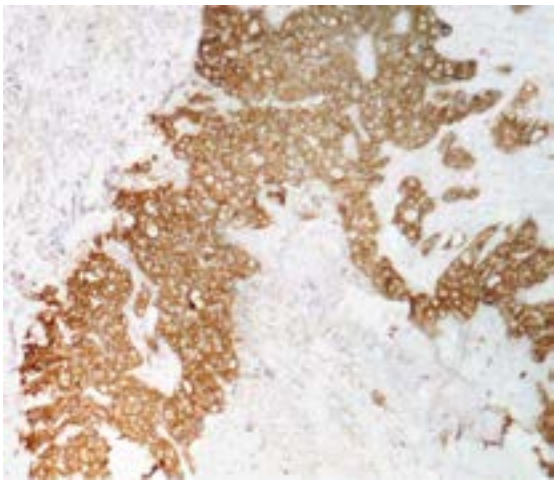

Fig. nr.53 Subtip molecular HER2pozitiv – forma histopatologică de carcinom ce celule clare. Imunomarcaj intens pozitiv pentru HER2 neu – scor 3+ (IHC 10x, caz nr.236, original)

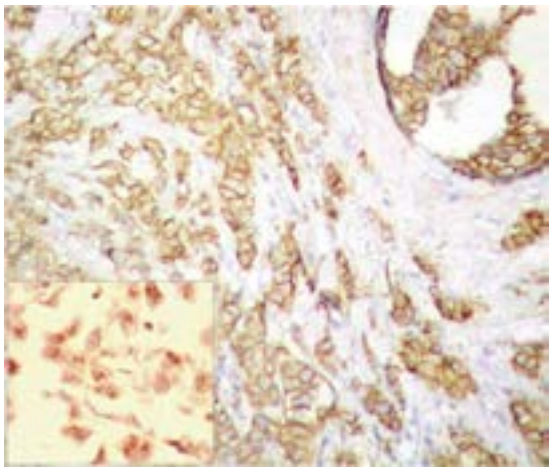

Fig. nr.54 HERpozitive molecular subtype - infiltrating apocrine carcinoma. IHC score 2+ for HER2neu; in box – the amplification of *HER2neu* gene (IHC 4x; in box - CISH 20x case no.177, original)

IX..5. Evaluation of TNP molecular subtype of invasive breast carcinoma

Breast cancer - TNP molecular subtype, the immunohistochemistry correspondent for basal-like intrinsic molecular subtype, is one of the most controversial classes of molecular classification and is associated with a great interest of researchers, because of its great heterogeneity at transcriptomic and genetic changes, which are more numerous than in other molecular subtypes [11,12]. Histopathological evaluation of thse cases revealed a high frequency of CDI-NST, recorded with 48,6% of cases. In the same time, it was observed that all cases of our study classified as metaplastic carcinoma (7 cases), medullary carcinoma (6 cases), adenoid – chistic carcinoma (3cases) and secretory carcinoma (1 case) were included in this molecular subtype (Fig. nr. 55- 58).

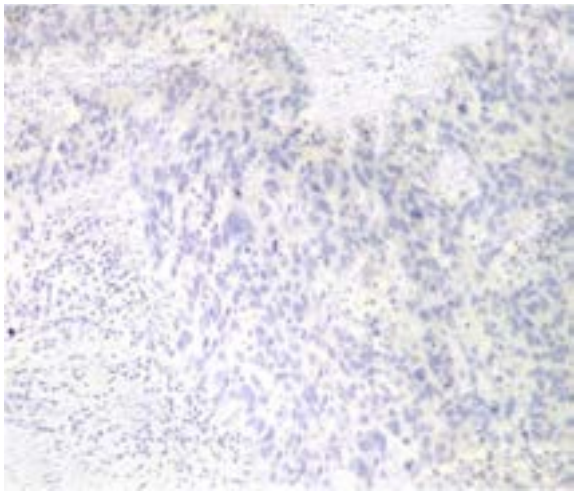

Fig. nr.55 TNP molecular subtype - invasive medullary carcinoma: negative imunostain for HER2 neu (IHC 10x case no.289, original)

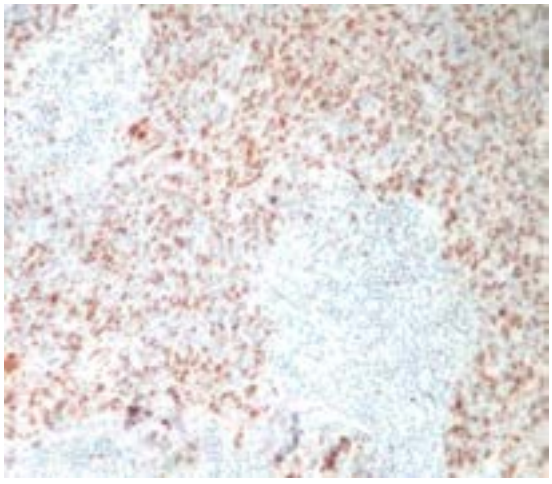

Fig. nr.56 TNP molecular subtype – invasive medullary carcinoma: strongly positive nuclear immunostain for p53 (IHC 10x case no.289, original)

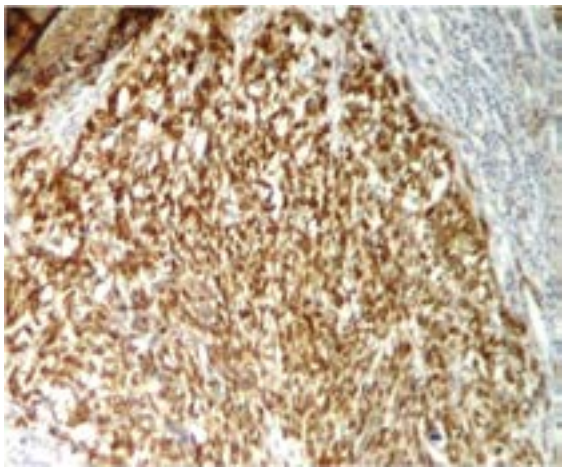

Fig. nr. 57 174 TNP molecular subtype – metaplastic carcinoma with fusiform cells: strongly positive immunostain for p53 (IHC 10x nr.287 case, original)

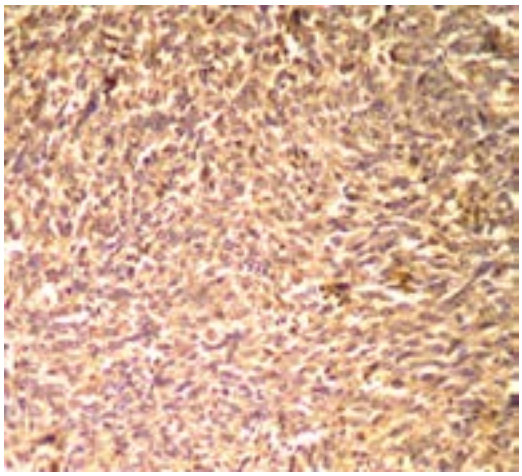

Fig. nr.58 TNP molecular subtype - metaplastic carcinoma with fusiform cells: positive immunostain for CK5 / 6 (IHC 10x, case no. 287, original)

IX.6.1. Correlations between molecular subtypes and main clinico-pathologycal and immunohistochemical factors

Molecular taxonomy of BC provides important information on the mechanisms of carcinogenesis involved in the onset, progression, metastasis and resistance to treatment, the prognostic and predictive value being validated and requiring prospective and retrospective studies that include representative samples of the population. Using immunohistochemical surrogate markers has facilitated the analysis of molecular subtypes for a larger number of cases than the one used in the first study. Therefore, a first step to understanding these molecular subtypes is to establish correlations with clinico-morphological and immunohistochemical aspects specific to each subtype. The immunohistochemical evaluation of the four main antibodies (ER, PR, Ki67 and HER2neu) was performed for 285 female patients, luminal subtype A is predominant with 43.6% followed by luminal B subtype with a percentage of 31.6% and TNP subtype by 12.3%, the correlations of the main factors clinico-morphological and immunohistochemical molecular classes are given in Table No. 3.

Tabel nr. 3 Correlations between molecular subtypes and main clinico-pathological and immunohistochemical factors

	Luminal A *n (%)*	**Luminal B** *n (%)*	**HER2 pozitiv** *n (%)*	**TNP** *n (%)*	**All cases** *n(%)*	*p*
	132 (46,3)	**90** (31,6)	**28** (9,8)	**35** (12,3)	285	
Age (years)						
average ± DS	61.57±11,6	61.62±11.01	58.71±10,5	55.43±11.3	60.55±11,4	**0,022***
Range	28 – 87	35 – 83	33 – 72	32 – 82	28 - 87	
Age categories						
<50 ani	19 (14.4)	14 (15.6)	5 (17.9)	10 (28.6)	48 (16.8)	**0,071**&
50-69 ani	74 (56.1)	51 (56.7)	19 (67.9)	20 (57.1)	164 (57.4)	
≥70 ani	39 (29.5)	25 (27.8)	4 (14.3)	5 (14.3)	73 (25.6)	
Hormonal status n (%)						
Premenopauză	21 (15.9	16 (17.8)	6 (21.4)	14 (40.0)	57 (20.0)	**0,026**§
Postmenopauză	111 (84.1)	74 (82.2)	22 (78.6)	21 (60.0)	228 (80.0)	
Maxim of tumoral diameter (cm)						
Average ±SD	2.38±1.5	2.90±1,8	3.21±2.0	3.82±2.5	2.80±1,9	**0,0001***

	Luminal A *n (%)*	Luminal B *n (%)*	HER2 pozitiv *n (%)*	TNP *n (%)*	All cases *n(%)*	*p*
	132 (46,3)	**90** (31,6)	**28** (9,8)	**35** (12,3)	285	
range	1 - 10	1 - 9	1 - 11	1 -14	1 - 14	
<2cm	25 (18.9)	11 (12.2)	2 (7.1)	2 (5.7)	40 (14.0)	
2 – 5cm	99 (75.0)	63 (70.0)	22 (78.6)	26 (74.3)	210 (73.7)	**0,0007**[&]
≥5 cm	8 (6.1)	16 (17,8)	4 (14,3)	7 (20.0)	35 (12.3)	
Laterality						
Left	63 (47,7)	44 (48,9)	15 (53,6)	15 (42,9)	137 (48,1)	
Right	68 (51,5)	46 (51,1)	13 (46,4)	20 (57,1)	147 (51,6)	**0,928**[§]
Bilateral	1 (0,8)	0 (0,0)	0 (0,0)	0 (0,0)	1 (0,4)	
Multicentricity/multifocality						
Yes	13 (9,8)	13 (14,4)	2 (7,1)	7 (20,0)	35 (12,3)	**0,291**[§]
No	119 (90,2)	77 (85,6)	26 (92,9)	28 (80,0)	250 (87,7)	
Localization						
CSE	82 (62,1)	57 (63,3)	12 (42,9)	15 (42,9)	166 (58,2)	
CSI	16 (12,1)	11 (29,7)	5 (17,9)	5 (14,3)	37 (13,0)	
CII	12 (9,1)	4 (4,4)	2 (7,1)	6 (17,1)	24 (8,4)	**0,171**[§]
CC	16 (12,1)	8 (8,9)	7 (25,0)	6 (17,1)	37 (13,0)	
CIE	3 (2,3)	5 (5,6)	1 (3,6)	0 (0,0)	9 (3,2)	
CE,CC,CI	3 (2,3)	5 (5,6)	1 (3,6)	3 (8,6)	12 (4,2)	
Histopatologic subtype						
CDI NST	82 (62,1)	68 (75,6)	27 (**96,4**)	16 (45,75)	193 (67,7)	
CLI	13 (9,8)	6 (6,7)	0	0	19 (6,7)	**0,0001**[§]
Ca mixt	10 (7,6)	7 (7,8)	1 (3,6)	1 (2,9)	19 (6,7)	
Grup I	27 (**20,5**)	9 (10,0)	0	0	36 (12,6)	
Grup II	0	0	0	**18**	18 (100)	
Nottingam histopatological grade (Elston-Ellismodified)						
GI-GII	111 (**84,1**)	64 (**71,1**)	13(46.4)	8 (22,9)	196 (62,1)	**0,0001**[§]
G III	21 (15,9)	26 (28,9)	15 (**53,6**)	27 (**77,1**)	89 (31,2)	
Peritumoral limfovascular invasion						
Present	31 (23,5)	40 (44,4)	8 (28,6)	7 (20,0)	86 (30,2)	**0,004**[§]
Absent	101 (76,5)	50 (55,6)	20 (71,4)	28 (80,0)	199 (69,8)	
Tumoral necrosis						
Yes	8 (6,1)	21(23,3)	5 (17,9)	13 (37,1)	47 (16,5)	**0,0001**[§]
No	124 (93,9)	69 (76,7)	23 (82,1)	22 (62,4)	238 (83,5)	
Central fibrosis						
Present	1 (0,8)	4(4,4)	3(10,7)	8 (22,9)	17 (6,0)	**0,0001**[§]
Absent	131 (99,2)	86 (95,6)	25 (89,3)	27 (77,1)	268 (94,0)	
The intensity of the inflammatory infiltrate						
Absent	109 (82,6)	46 (51,1)	14 (50,0)	13 (37,1)	182 (64,1)	
Low	18 (13,6)	30 (33,3)	9 (32,1)	10 (28,6)	66 (23,2)	**0,0001**[&]
Moderate	4 (3,0)	3 (3,3)	1 (3,6)	1 (2,9)	9 (3,2)	
High	1 (0,8)	11 (12,2)	4 (14,3)	11 (31,4)	27 (9,5)	
Limfonodular status						
Positive	62 (52,5)	62 (73,8)	20 (76,9)	26 (83,9)	170 (65,6)	**0.005**[§]
negative	56 (47,5)	22 (26,2)	6 (23,1)	5 (16,1)	89 (34,4)	
absent	14	6	2	4	26	
pTstage						
pT1	72 (54,5)	31 (34,4)	7 (25,0)	5 (14,3)	115 (40,4)	
pT2	44 (33,3)	29 (32,2)	12 (42,9)	15 (42,9)	100 (35,1)	**0,0001**[&]
pT3	4 (3,0)	4 (4,4)	0	3 (8,6)	11 (3,9)	
pT4	12 (9,1)	26 (28,9)	9 (32,1)	12 (34,3)	59 (20,7)	
pN stage						
pN0	60 (50,5)	22 (26,2)	5 (19,2)	5 (16,1)	92 (35,2)	**0,0001**[&]
pN1	36 (30,0)	18 (21,4)	5 (19,2)	5 (16,1)	64 (24,5)	

	Luminal A *n (%)*	Luminal B *n (%)*	HER2 pozitiv *n (%)*	TNP *n (%)*	All cases *n(%)*	*p*
	132 (46,3)	**90** (31,6)	**28** (9,8)	**35** (12,3)	285	
pN2	21 (17,5)	28 (33,3)	8 (30,8)	13 (41,9)	70 (26,8)	
pN3	3 (2,5)	16 (19,0)	8 (30,8)	8 (25,8)	35 (13,4)	
pNx	12	6	2	4	24	
pTNM stage						
I	44 (36,7)	14 (16,3)	3 (11,5)	2 (6,1)	63 (23,8)	
II	46 (38,3)	22 (26,7)	4 (15,4)	8 (21,2)	80 (30,2)	**0,0001**[&]
III	30 (25,0)	37 (41,9)	17 (65,4)	14 (45,5)	98 (37,0)	
IV	0	13(15,1)	2 (7,7)	9 (27,3)	24 (9,1)	
pTNMx	12	4	2	2	20	
Associted in situ carcinoma						
Prezent	93 (70,5)	58 (64,4)	18 (64,3)	15 (42,9)	184 (64,6)	**0,027**[§]
Absent	39 (29,5	32 (35,6)	10 (35,7)	20 (57,1	101 (35,4)	
PR Status						
Negative	0 (0,0)	10(11,1)	28 (100)	35 (100)	73 (25,6)	**0,0001**[&]
Positive 1-10%	2 (1,5)	34 (37,8)	0 (0,0)	0 (0,0)	36 (12,6)	
Positive ≥ 10%	130 (98,5)	46 (51,1)	0 (0,0)	0 (0,0)	176 (61,8)	
ER Status						
Negative	0(0,0)	1(1,1)	28(100)	35(100)	64(22,5)	
Positive 1-10%	5(3,8)	27 (30,0)	0(0,0)	0(0,0)	32 (11,2)	**0,0001**[&]
Positive ≥ 10%	127 (96,2)	62 (68,9)	0(0,0)	0(0,0)	189 (66,3)	
Immunostain/amplification of HER2neu						
Positive	0 (0,0)	57 (63,3)	28 (100)	0 (0,0)	85 (29,8)	**0,0001**[§]
Negative	132 (100)	33 (36,7)	0 (0,0)	35 (100)	200 (70,2)	
Ki67 Index						
Negative	17 (12,9)	10 (11,1)	2 (7,1)	2 (5,7)	31 (10,2)	
Positive <14%	115 (87,1)	28 (31,1)	12 (42,9)	3 (8,6)	158 (55,4)	**0,0001**[§]
Positive ≥14%	0 (0,0)	52 (57,8)	14 (50,0)	30 (85,7)	96 (33,7)	
Imunomarcaj P53						
Negativ	61 (75,3)	49 (64,5)	10 (41,7)	1 (3,0)	121 (56,5)	
Pozitiv	20 (24,7)	27 (35,5)	14 (58,3)	32 (97,0)	93 (43,5)	**0,0001**[§]
Absent	51	14	4	2	71	
EGFR immunostain						
Positive	10 (20,0)	8 (20,0)	11 (52,4)	21(72,4)	50(35,7)	**0,0001**[§]
Negative	40 (80,0)	32 (80,0)	10 (47,6)	7 (27,6)	90 (64,3)	
Absent	82	50	7	6	140	
CK5/6 immunostain						
Positive	11 (23,9)	12 (30,0)	6 (33,3)	21 (77,8)	50 (38,2)	
Negative	35 (76,1)	28 (70,0)	12 (66,7)	6 (22,2)	81 (61,8)	**0,0001**[§]
Absent	86	50	10	8	154	

[*] - ANOVA test; [&] -Kruskal-Wallis test; [§] - Chi squre test

IX. 6.2. Survival and molecular subtype

Overall survival (OS) was performed for 239 cases that belonged to females, diagnosed within 5 years and a mean follow-up of 42 months (range 15-74). In this group of patients 9.6% of patients died of BC, and the distribution of molecular subtypes was represented by 46.9% for luminal A, 29.7% for luminal B (10.9% luminalB-HER2negative and 8% for luminal B-HER2positive), 10.5% for HER2positive and 13.0% for TNP molecular subtype. This distribution of cases is similar to that obtained in the main lot of patients, with no statistically significant differences.

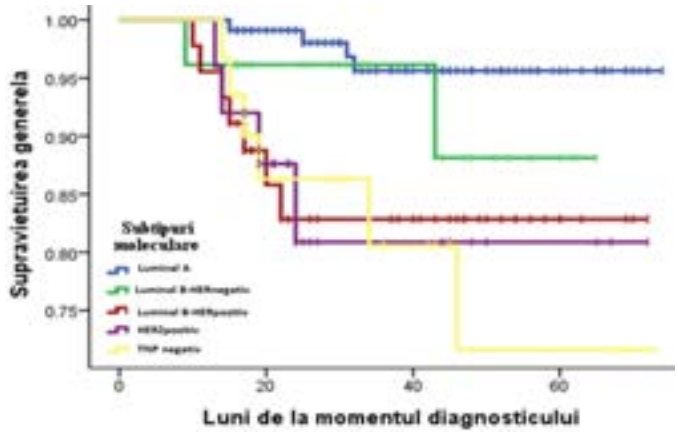

Fig. nr. 59 Kaplan-Meier survival curve for the molecular subtypes

OS for all cases evaluated established an 90.4%, overall survival rate for the entire group of patients, and for each molecular subtype separately were obtained following survival rates: 96.4% - luminal A, 92, 3% - luminal B-HER2negative, 84.4% - luminalB-HER2positive, 84.0% - 80.6% for HER2positive and TNP. It is noted that the TNP molecular subtype has the lowest survival rate, followed by HER2 status subtypes (HER2-positive molecular subtype and Luminal B - HERpositive) and the best survival rate was observed for cases assigned as Luminal A molecular subtype (Fig. no. 59), these differences being statistically significant (Log Rank test, p = 0, 01). Among the clinico -morphological factors influencing an unfavorable prognosis in our sample of patients with a statistically significant impact stands the maximum tumor diameter of more than 2 cm, BC poorly differentiated, regional lymphatic metastases in more than three nodules, presence of peritumoral lymphovascular invasion, advanced disease, p53 oncoprotein expression and a high rate of nuclear proliferation objectified by a high Ki67 index.

CHAPTER X - CONCLUSIONS

Clinico-statistical study was conducted on a sample of 293 patients diagnosed with invasive breast carcinoma and a complete immunohistochemical examination consisting of four biomarkers (ER, PR, HER2neu, Ki67) during the period of 2008 to 2013, which was completed by the following conclusions:

1. **Distribution of cases** according to years of study noted a steady increase with a "peak" of the number of new cases diagnosed and analyzed for 2012, the female cases dominating the spectrum of malignant lesions (97.3%) compared with male gender which was accounted with only 8 cases.
2. **Morphological analysis** revealed the predominance of cases with a diagnosis of invasive ductal carcinoma CDI-NST with 67.6%, the second place being represented by the mixed forms of invasive breast carcinoma with a percentage of 7.2% of cases. The difference was represented by the special or rare forms of invasive breast carcinoma, among which stands invasive lobular carcinoma with the largest share - 19cazuri (6.5%). Moderately differentiated forms have the highest percentage of 58%, and association with an in situ component was observed in 57.1% of cases.
3. **Immunohistochemical analysis** allowed the identification of hormone receptor expression with a frequency of 78.2% for ER and 74.7% for PR. HER2neu oncoprotein overexpression was observed in 20.8% of cases, and an equivocal score was obtained for 16.0% of cases. Quantifying the rate of proliferation by assessing the immunohistochemical expression of Ki67 a high index was observed in 31.1%, of which 56.0% of the cases are presented forms with low-differentiated

histopathology. A positive immunostain for p53 oncoprotein was obtained in 33.1%, more than 70% of them in association with low histopathological grade, tumor diameter over 5 cm and a positive lymphatic status.

4. **Amplification of HER2/neu** was evaluated for 47 cases by chromogenic in situ hybridization, showing a high level of amplification in 40.4% of cases and a low level for 17.0% cases. Overall, the status HER2pozitive was identified in 88 cases, between the two methods being a good correlation.

5. **Molecular classification** based on immunohistochemical "surrogate" markers showed an increased frequency of luminal A subtype with a 46.1% frequency, followed by the luminal B with a frequency of 32.4%. The difference was represented by TNP molecular subtype with 11.9% and HER2positive molecular subtype with 9.6% of cases, all of them belonged to female cases, in contrast to the male cases which were identified only in luminal molecular subtypes.

6. For **Luminal A molecular subtype** the mean age at diagnosis was 61.5 years, the highest percentage of cases being found in the 5th and 6th decade and with a 2,2cm mean tumor diameter. Histopathological form of CDI-NST was recorded with a 62.1% frequency, the rest being characterized by specific forms of BC, the majority of them being CLI (9.6%) or mucinous carcinoma (6.8%). All cases with neuroendocrine carcinoma diagnosis (2 cases), cribriform carcinoma (10 cases) and invasive tubular carcinoma (3 cases), which are associated with a good prognosis, were included in this molecular subtype. The association with an in situ component was recorded with a high percentage 68.9% and peritumoral lymphovascular invasion was observed in 38.6% of cases. Nodular status was negative in 45.3% of cases and 27.5% were classified as pN1. P53 oncoprotein expression was obtained in 17 cases, of which 52.9% of cases were moderately differentiated forms and 41.2% poorly differentiated.

7. **Luminal B molecular subtype** represent a heterogeneous group of breast carcinoma consisting of 95 cases: luminal B-HER2 negative was recorded with a frequency of 11.9% of all cases included in the study, and luminal B-HERpositive for 20.9% of cases. Mean age of patients in this molecular subtype is similar to luminal subtype A and the mean tumor diameter was 2,8cm, without identifying a significant differences between the two forms of molecular subtype. Rare histopathological forms assigned to this subtype are represented by micropapillary carcinoma (2.1%), CLI (6.3%) and intraductal papillary carcinoma associated with CDI-NST (6.4%). Histopathological forms were mostly moderately differentiated, and the presence of metastases in more than four regional lymph nodes was observed at a rate of 70.8% which is associatated with bad prognosis. In terms of tumor stage the highest percentage (44%) was obtained for stage IV, followed by stage II, with a 24% frequency. Quantification of p53 oncoprotein expression led to statistical significantly differences between the two subgroups, luminal B subtype - HER2positive having the largest number of cases (24) with a positive expression.

8. **HER2 positive molecular subtype** was characterized by a mean tumor diameter of 3.11 cm, higher than luminal subtypes, with a preponderance of cases for the sixth decade. In this molecular subtype, from special histopathological forms were observed all cases of Paget's disease associated with CDI-NST (3 cases), apocrine carcinoma (one case) and clear cell carcinoma (one case). From the point of view of histopathological grade, the poorly differentiation forms predominated (57.1%) and stage III tumor was the most frequent (60.7%). Overexpression of p53 oncoprotein was obtained at a rate of 58.3% and in 50% of cases was observed a high Ki67 index.

9. **TNP molecular subtype** was characterized by an increased frequency of cases in the 4th decade of life, most of them (74.3%) having a mean diameter over 2 cm and 77.1% were represented by poorly differentiated histopathological forms. Special histopathological forms that

were classified in this category are represented by metaplastic carcinoma (7 cases), medullary carcinoma (6 cases), adenoid-cystic carcinoma (3 cases) and secretory carcinoma (one case). Of significant morphological characteristics of TNP molecular subtype were noted: tumoral necrosis of geographic type, poorly differentiated tumors with a syncytial pattern, abundant peri- and intratumoral inflammatory infiltrate and central fibrosis. In terms of immunophenotype, this molecular subtype was characterized by increased expression of the oncoprotein p53 and Ki67 index. Further analysis of basal biomarkers (EGFR, CK5/6) found their expression in a relatively similar percentage (37.4% versus 36.6%), which allowed a better identification of these cases.

10. **Establishing correlations** between molecular subtypes and the main pathological features revealed a highly statistically significant differences ($p \leq 0, 0001$) with the following features: the mean tumor diameter, histopathological subtype, morphological grade, peritumoral limfovascular invasion, Ki67 index, immunohistochemical expression of EGFR and CK5/6. Were also observed molecular differences between classes and the hormonal status ($p = 0.026$), mean age ($p = 0.045$) lymphatic status ($p = 0.005$) and the association with in situ component ($p = 0.027$).

11. **Analysis of short term overall survival** (OS) was performed for 239 patients with a median follow-up of 42 months (range 15 months - 74 months,) for the entire sample yielding a survival rate of 90.4%. Also, it was noted a reduced survival rate for TNP molecular subtype with 80.6% and the best rate was identified in the luminal A subtype (92.3%) and luminal B molecular subtype HERnegativ 92.3%. With approximately equal survival rates, 84.4% and 84.0% were obtained for luminalB-HER2positive molecular subtype, respectively HER2positive.These differences were shown to be statistically significant ($p = 0, 01$).

12. **This study, which gathered complex pathological, immunohistochemical and molecular data of invasive breast carcinomas on a representative population sample, prooves the great heterogeneity of breast carcinomas and demonstrates that molecular classification based on the quantification of four biomarkers (ER, PR, Ki-67, HER2neu) has an important prognostic role, being complementary with the main clinico-morphological and immunohistochemical factors.**

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