

Assessment of hormonal receptor immunoexpression and Her2 status in invasive breast cancer after conservative and radical surgery

TIBERIU ȘTEFĂNIȚĂ ȚINEA-COJAN¹⁾, ADRIAN MACOVEI²⁾, ION PĂUN¹⁾, ANDREI IONUȚ COSTIN¹⁾, CLAUDIA-VALENTINA GEORGESCU³⁾, CORNELIU CRISTIAN GEORGESCU⁴⁾, IONELA MIHAELA VLADU⁵⁾, CRISTINA GABRIELA ENE⁶⁾, LUCREȚIU RADU⁷⁾

¹⁾Department of Surgery, University of Medicine and Pharmacy of Craiova, Romania

²⁾Department of Extreme Conditions Medicine, University of Medicine and Pharmacy of Craiova, Romania

³⁾Department of Pathology, Emergency County Hospital, Craiova, Romania

⁴⁾Department of Pharmacology, University of Medicine and Pharmacy of Craiova, Romania

⁵⁾Department of Diabetes and Metabolic Diseases, University of Medicine and Pharmacy of Craiova, Romania

⁶⁾PhD Student, University of Medicine and Pharmacy of Craiova, Romania

⁷⁾Department of Hygiene, University of Medicine and Pharmacy of Craiova, Romania

Abstract

This study was conducted on 300 patients with infiltrative breast carcinoma, of which 112 cases underwent radical mastectomy, and 188 were conservatively operated. Forty-four patients experienced local relapses during the clinical follow-up. The immunohistochemical (IHC) study used the Labeled Streptavidin Biotin (LSAB)/Horseradish peroxidase (HRP) method, which evaluated the estrogen receptor (ER) and progesterone receptor (PR), as well as human epidermal growth factor receptor 2 (Her2/neu) expressions in tumor cells. After IHC analysis, all cases were classified into one of the following four immunophenotypes: ER+/PR+ classical phenotype, ER-/PR-, ER+/PR- and ER-/PR+ phenotypes. Correlating hormonal phenotypes with Her2 status, we found that Her2 is significantly associated more frequently with the ER+/PR- phenotype, while the absence of Her2 was associated more frequently with the ER+/PR+ phenotype but with no significant differences. Local recurrences were significantly more common in patients with absent ERs than those who had these receptors present in the primary tumor. Similarly, mammary tumors with absent PRs recurred significantly more frequently than those with PRs. The positivity of Her2 is significantly associated with the absence of PRs in both type of surgery (conservative or radical). Local recurrences are significantly correlated with both negative receptors phenotype, regardless of the type of surgery. IHC markers, represented by hormone receptors for estrogen and progesterone, and Her2 oncoprotein, can be useful for identifying patients who are at increased risk of local recurrences after conservative or radical surgery for breast cancer.

Keywords: breast cancer, conservative surgery, hormonal receptor, mammary carcinoma.

Introduction

Breast cancer is one of the modern world scares. There are more than 10 million cases per year. It accounts for 25% of the cancer cases and 15% of the cancer deaths. Its highest prevalence remains in the 55–64 years old group [1].

With all the medical and technical progress, the current management guidelines still depend heavily on histological types, especially on estrogen and progesterone hormone receptors, as well as the expression of human epidermal growth factor receptor 2 (Her2) [2]. Unfortunately, no matter how well these histological types are classified and used in a sufficient number of good studies, the clinical outcome of breast cancer still appears to have a mind of its own. There is hope that, in the future, the increased availability of fast gene sequencing machines will bring new insides in this critical oncological area [3].

The classical pattern of breast cancers includes two main groups, based on estrogen receptor (ER) expression.

The ER+ types, depending on various protein expressions on the luminal side are grossly classified in at least two groups, luminal A and B, with debate still going on. Clinically more significant, the ER- groups have been studied with dedicated attention and classified as Her2+, basal like tumors and the so-called “normal” breast-like tumors [4].

Further attempts were made to differentiate luminal cells, by using cytokeratins expressions (CK7, CK8, CK18, CK19), mucin 1 (MUC1), $\alpha 6$ integrin, B-cell lymphoma 2 (BCL2), ER, progesterone receptor (PR), GATA3 and adhesion molecules [5].

Coming closer to the aim of this paper, Her2 is expressed in 18–20% of all breast cancers. The official gene name is *ERBB2*, although due to studies interchangeability reasons most authorities opt for keeping Her2 designation. Her2 expression has been clinically correlated with breast cancer outcomes, in the aspect that Her2 expression warrants a darker prognosis. It is a perhaps the most relevant (definitely the most known)

oncological marker to warrant a systemic therapy on breast cancer [6, 7]. Her2 expression might be used as therapeutic agent indicator, being associated with some resistance to Tamoxifen or certain chemotherapeutic agents (non-anthracyclines) [8]. Even more important, new therapies that aim for Her2 sites (like the Trastuzumab antibody) have been associated with improved outcomes [9].

Research on breast cancer stem cells (BCSCs) has identified some markers [hyaluronan receptor (CD44), signal transducer CD24 and aldehyde dehydrogenase-1 (ALDH-1)], which were associated with poor prognosis and resistance to usual therapies [10].

Triple negative tumors have long been a dreaded clinical encounter. Although triple negative may not be entirely accurate histologically speaking, a cell type that lacks ER, PR or Her2 expression is associated with poor outcome [11].

It is this variability of outcomes with various markers that pushed towards aim of this paper. Other studies have focused on similar goals as ours (Nguyen *et al.* study [12] but aimed towards a narrower therapeutic array. The Kyndi *et al.* study [13] aimed for the same goal in the context of radical mastectomy and radiotherapy. It is expected that the results of this study to be a valuable aid for all practitioners involved in breast cancer management.

Aim

For the first time in this geographical area, we aimed for a prognosis association between the histological type of the breast cancer and the outcome following both conservative and radical surgery.

Materials and Methods

The studied material was represented by fragments of tumoral mammary tissue, which were harvested from patients with an invasive mammary carcinoma underwent

a conservative or a radical surgery, in two University Clinics in Craiova (Romania): the Surgical Clinic IV of the Railroads Hospital and the Surgical Clinic I of the Emergency County Hospital, over a period of 18 years, between 1995 and 2012. Out of the 1358 cases of infiltrating mammary carcinomas operated on over this period of time, 502 cases underwent conservative surgeries and 856 cases underwent radical surgeries. The immunohistochemical (IHC) study included a number of 300 patients who were represented by the group of patients which underwent conservative surgeries, represented by 112 cases with infiltrating mammary carcinomas, and by the group of patients which underwent radical surgeries, represented by 188 cases with infiltrating mammary carcinomas. Out of the total of immunohistochemically-analyzed cases, 44 cases presented local recurrences (25 cases with infiltrating tumors, which underwent conservative surgeries, and 19 cases with infiltrating tumors, which underwent radical surgeries recurred at the local level). The IHC study was done at the Laboratory of Histology and Immunohistochemistry of the University of Medicine and Pharmacy of Craiova.

Immunohistochemistry

The IHC method was based on immunoenzymatic soluble complexes, called Labeled Streptavidin Biotin (LSAB)/Horseradish peroxidase (HRP). The set we used was Universal DAKO LSAB 2 System/HRP. There were used concentrated antibodies produced by DAKO Cytomation Company, Denmark, whose dilutions and pre-treatments are presented in Table 1. In order to obtain the best dilution, the antibodies were dissolved in phosphate-buffered saline (PBS)-azide, at the moment of utilization. From the paraffin blocks analyzed through IHC for Her2 that received an equivocal 2+ score, we extracted 4–5 μ m thick sections that were exposed on SuperFrost™ Plus slides and these sections were subject to chromogenic *in situ* hybridization (CISH) exam.

Table 1 – Used antibodies, clones, dilutions and the pre-treatment

Antibody	Clone	Dilution	Pre-treatment	Incubation time	External control
ER	1D5	1:50	EDTA solution, pH 8, 20 minutes at microwaves	One hour at 37°C	Mammary carcinoma
PR	PgR 636	1:50	EDTA solution, pH 8, 20 minutes at microwaves	One hour at 37°C	Mammary carcinoma
Her2	Polyclonal	1:250	Citrate solution, pH 6, 20 minutes at microwaves	30 minutes at room temperature	Mammary carcinoma

ER: Estrogen receptor; PR: Progesterone receptor; Her2: Human epidermal growth factor receptor 2; EDTA: Ethylenediaminetetraacetic acid.

For the interpretation of the results for the Her2 immunostaining, we used the *American Society of Clinical Oncology* (ASCO)/*College of American Pathologists* (CAP) recommended criteria (2007) [14]. To quantify the status of the hormonal receptors, we used the Allred score [15]. Tumors with an Allred score ≤ 2 were considered negative and those with an Allred score above 2 were thought of as positive.

The cases evaluated through the CISH method were analyzed as follows:

- Amplification (high or low) – result was CISH Her2 positive. Tumors with a high amplification of the *HER2* gene presented more than 10 points or big clusters, or multiple points and big clusters that were seen in more than 50% of the nuclei of the tumor cells for the area chosen as significant for analyzing. The tumor with a low

amplification of the *HER2* gene presented more than 5 but less than 10 points or small clusters, or multiple points and small clusters in more than 50% on the nuclei of the tumor cells analyzed.

- Lack of amplification – result was CISH Her2 negative. These were either tumors with polysomy of the chromosome 17: presenting with 3–5 points/nuclei in more than 50% of all the tumor cells from the selected area; or diploid tumors with normal status for the *HER2* gene with 1–2 points/nuclei in more than 50% of all tumor cells from the selected area.

Results

A number of 112 cases of invasive carcinoma, which underwent a conservative surgery, and 118 cases of

invasive carcinoma, which underwent a radical surgery, have been analyzed. Altogether, a number of 300 cases were processed immunohistochemically, with a view to determine the expression of the hormone receptors and Her2. We need to mention that in this group, which was immunohistochemically analyzed, there were also included 25 cases of invasive mammary carcinomas, which recurred ipsilaterally after the conservative surgery, as well as the 19 cases which underwent radical surgeries and presented local recurrences.

Evaluation of hormone receptors (ER, PR) expression

The analysis of the immunomarkers to the hormone receptors (ER and PR) allowed qualifying the analyzed tumors as immunophenotypes which we later reported to the status of immunohistochemically detected Her2 protein. Subsequently, the cases with ipsilateral recurrences were analyzed separately from the point of view of the hormone receptors expression and Her2 status.

The analysis of ER immunostaining highlighted the presence of the ERs (Figure 1) in 80 of the 112 (71.42%) cases which underwent conservative surgeries, and in 136 of the 188 (72.34%) cases which underwent radical surgeries. The rest was composed of 32 (28.58%) cases of the ones which underwent conservative surgeries for

ER (Figure 2), and also 52 (27.66%) cases of the ones which underwent radical surgeries. Similarly, we followed the presence of PR immunomarkers and noticed results, which are similar to ER immunomarkers. Thus, 76 (67.86%) cases were PR positive (Figure 3) and 36 (32.14%) cases were PR negative (Figure 4), for the cases which underwent conservative surgeries, and 129 (68.61%) cases were PR positive, while 59 (31.39%) cases were PR negative, for the cases with radical mastectomy (Table 2). The statistical analysis did not show significant differences regarding the positivity rate of ER and PR in the number of people who underwent conservative surgeries, in comparison with the ones who underwent conservative surgeries ($p=0.864$ and $p=0.89$, respectively).

Afterwards, each of the 300 cases were classified according to one of the following four immunophenotypes: the ER+/PR+ classical phenotype (70 cases which underwent conservative surgeries and 119 cases which underwent radical surgeries); the ER-/PR- phenotype (25 cases which underwent conservative surgeries and 41 cases which underwent radical surgeries); the ER+/PR- phenotype (11 cases which underwent conservative surgeries and 18 cases which underwent radical surgeries); and the ER-/PR+ phenotype (six cases which underwent conservative surgeries and 10 cases which underwent radical surgeries).

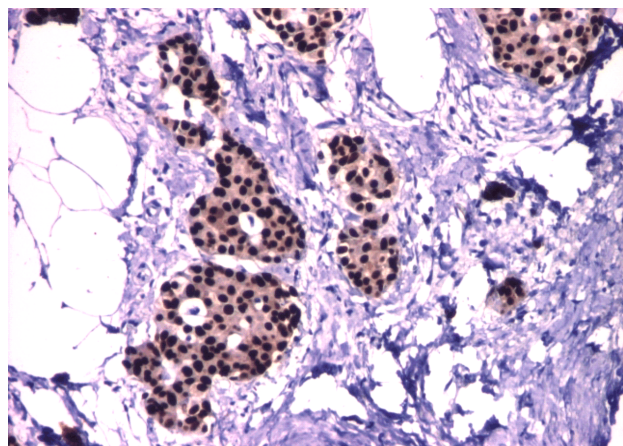


Figure 1 – Positive immunomarker for estrogen receptor (ER) in tumoral cells (infiltrating mammary carcinoma). Anti-ER antibody immunostaining, $\times 100$.

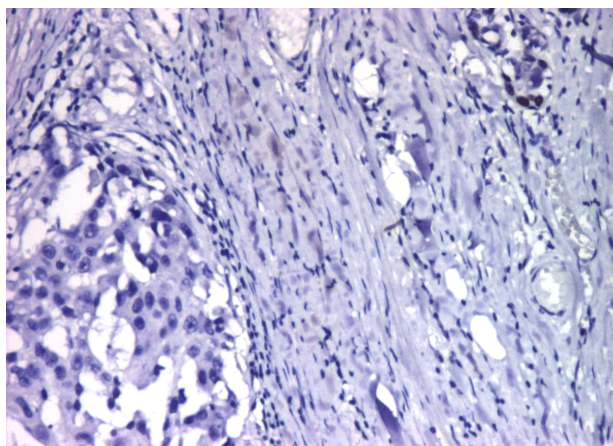


Figure 2 – Negative immunomarker for estrogen receptor (ER) in tumoral cells, internal positive control (normal ducts and acini). Anti-ER antibody immunostaining, $\times 100$.

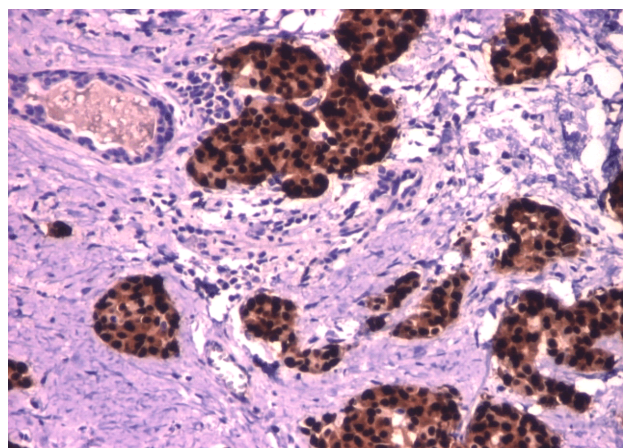


Figure 3 – Positive immunomarker for progesterone receptor (PR) in tumoral cells (infiltrating mammary carcinoma). Anti-PR antibody immunostaining, $\times 100$.

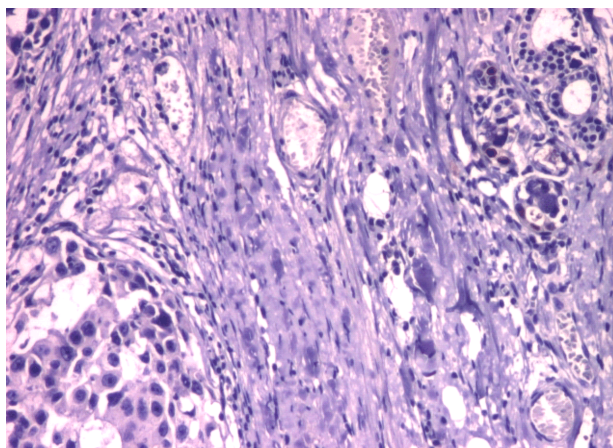


Figure 4 – Negative immunomarker for progesterone receptor (PR) in tumoral cells, internal positive control (normal ducts and acini). Anti-PR antibody immunostaining, $\times 100$.

Table 2 – The distribution of the cases according to the ER and PR immunomarkers

	ER+		ER-		PR+		PR-	
	C	R	C	R	C	R	C	R
No. of cases	80	136	32	52	76	129	36	59
Percentage	71.42%	72.34%	28.58%	27.66%	67.86%	68.61%	32.14%	31.39%

ER: Estrogen receptor; PR: Progesterone receptor; C: Conservative; R: Radical.

The hormone immunophenotype which was the most frequently met was the classical one with both hormone receptors present (ER+/PR+), both for the cases which underwent conservative surgeries (70 cases, 63.41%), and in the case of patients with radical mastectomy (119 cases, 63.3%). In the immunophenotype with both negative hormone receptors (ER-/PR-), there were 25 (21.95%) cases

which underwent conservative surgeries and 41 (21.81%) cases after the radical surgeries, while the immunophenotypes with only one positive hormone receptor (ER+/PR- and ER-/PR+) included the fewest cases, being represented by 11 (9.82%) and six (5.36%) cases for conservative surgeries and 18 (9.57%) along with 10 (5.32%) cases for radical surgeries (Table 3; Figure 5).

Table 3 – The frequency of hormone immunophenotypes

	ER+/PR+		ER-/PR-		ER+/PR-		ER-/PR+	
	C	R	C	R	C	R	C	R
No. of cases	70	119	25	41	11	18	6	10
Percentage	62.5%	63.3%	22.32%	21.81%	9.82%	9.57%	5.36%	5.32%

ER: Estrogen receptor; PR: Progesterone receptor; C: Conservative; R: Radical.

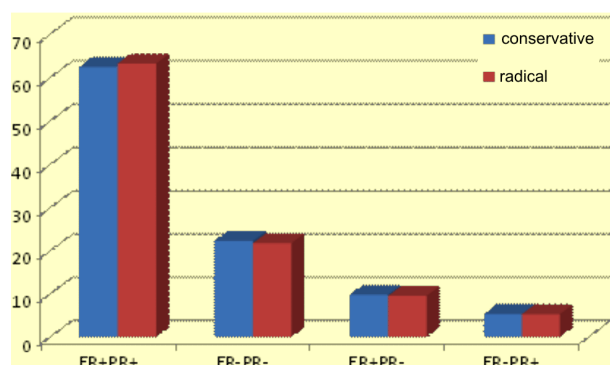


Figure 5 – Distribution of cases according to the immunophenotypes of hormone receptors. ER: Estrogen receptor; PR: Progesterone receptor.

Evaluation of the status of Her2/neu protein

The interpretation of the immunomarker for Her2 oncoprotein was done according to the ASCO/CAP recommendations, the cases being classified as follows: 0 (negative), 1+ (negative), 2+ (slightly positive – doubtful) and 3+ (positive). In Table 4, we presented the classification according to the Her2 score amongst the 112 mammary carcinomas that underwent conservative surgeries.

Table 4 – The distribution of the cases which underwent conservative surgeries according to Her2/neu score

	Her2/neu Score 0	Her2/neu Score 1+	Her2/neu Score 2+	Her2/neu Score 3+
No. of cases	46	38	22	6
Percentage	41.08%	33.92%	19.64%	5.36%

Her2/neu: Human epidermal growth factor receptor 2.

The cases with Her2 2+ (score) immunomarker were borderline (equivocal) from the point of view of Her2 positivity and had to undergo further examination, which is required in such cases. Therefore, these cases were studied through CISH. After the CISH examination of the 22 cases with Her2 2+ equivocal immunomarker, which underwent conservative surgeries, it was found that nine cases presented the amplification of *HER2* gene

and were qualified as positive, while 13 cases were non-amplified and were qualified as negative. Thus, in the group of the patients who underwent conservative surgeries, there were 15 cases with *HER2*+ status after the CISH examination (six cases with IHC 3+ score and nine cases with IHC 2+ score and the amplification of *HER2* gene). Similarly, the 38 cases which underwent radical surgeries with Her2 2+ immunomarker were separated after the CISH examination in 15 cases with the amplification of *HER2* gene and 23 non-amplified cases. Consequently, in the group of the patients who underwent radical surgeries, there were 24 cases with *HER2*+ status (nine cases with IHC 3+ score and 15 cases with IHC 2+ score and the amplification of *HER2* gene) (Table 5).

Table 5 – The distribution of the cases which underwent radical surgeries according to Her2/neu score

	Her2/neu Score 0	Her2/neu Score 1+	Her2/neu Score 2+	Her2/neu Score 3+
No. of cases	77	64	38	9
Percentage	40.96%	34.04%	20.21%	4.79%

Her2/neu: Human epidermal growth factor receptor 2.

After the definitive determination of Her2 status by means of IHC analysis (Figures 6–9), combined with the CISH examination, there were obtained the correlations of *HER2* status with the four immunophenotypes for the groups of patients who underwent conservative surgeries (Table 6) or radical surgeries (Table 7).

In relation to the hormone phenotypes, we noticed that the tumors with both hormone positive receptors (ER+/PR+ phenotype) are more frequently *HER2*- (Table 7) than *HER2*+ [95.71% vs. 4.29% for the cases who underwent conservative surgeries (Figure 10) and 95.78% vs. 4.2% for the cases who underwent radical surgeries (Figure 11)]. On the other hand, the tumors lacking in the two hormone receptors (ER-/PR- phenotype) were *HER2*- in 76% of the cases who underwent conservative surgeries and in 78.05% of the cases who underwent radical surgeries (Figure 9), and *HER2*+ in 24% of the cases who underwent conservative surgeries and 21.95% of the cases who underwent radical surgeries (Figure 7).

The mammary carcinomas lacking in PR (ER+/PR- phenotype) were *HER2*- in 54.55% of the cases who underwent conservative surgeries and 55.56% of the cases which underwent radical surgeries, and the *HER2*+ ones were found 45.45% of the cases which underwent conservative treatments and 44.44% of the cases which underwent radical treatment (Figure 8), while the carcinomas lacking in ER (ER-/PR+ phenotype) were *HER2*- in 83.33% of the cases which underwent conservative surgeries and in 80% of the cases which underwent radical surgeries. Thus, we observed that *HER2* positivity is associated most frequently to the ER+/PR- phenotype (45.45% of the cases which underwent conservative surgeries and 44.44% of the cases which underwent radical surgeries), this association being significant from the statistical point of view both for the cases which underwent conservative surgeries ($p=0.0085$) and for the cases which underwent radical surgeries ($p=0.00028$). The absence of *HER2* was most frequently associated with the ER+/PR+ phenotype (95.71% of the conservative cases and 95.78% of the radical cases) (Figure 6), between the *HER2*- status and the hormone phenotypes not existing significant correlations from the statistical point of view ($p=0.758$ for the cases which underwent conservative

surgeries and $p=0.552$ for those which underwent radical surgeries).

Immunohistochemical analysis of the cases with recurrences

Out of the 502 cases of mammary carcinomas which underwent conservative surgeries, a number of 25 (5.18%) cases presented ipsilateral local recurrences. Out of the 856 tumors which underwent radical surgeries, a number of 19 (2.22%) cases recurred locally and were included in this IHC study.

The IHC analyzed local ipsilateral recurrences in the case of invasive mammary carcinomas which underwent conservative surgeries (Table 8) and radical surgeries (Table 9), respectively, were more frequent for ER- tumors in comparison with ER+ tumors [16 (50%) cases vs. nine (11.25%) cases for the recurrences after conservative surgeries; 12 (23.08%) cases vs. seven (5.15%) cases for the recurrences after radical surgeries]. The statistical analysis proved that the recurrences were more significantly frequent at the ER- patients, in comparison with patients having ER+ primary tumor ($p=0.0078$ for the conservative group and $p=0.0014$ for the radical group, respectively).

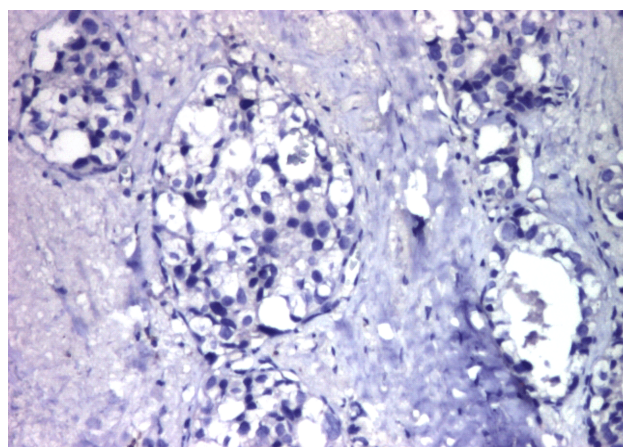


Figure 6 – Invasive mammary carcinoma: Her2 negative (0), ER+/PR+. Anti-Her2 antibody immunostaining, ×100. Her2: Human epidermal growth factor receptor 2; ER: Estrogen receptor; PR: Progesterone receptor.

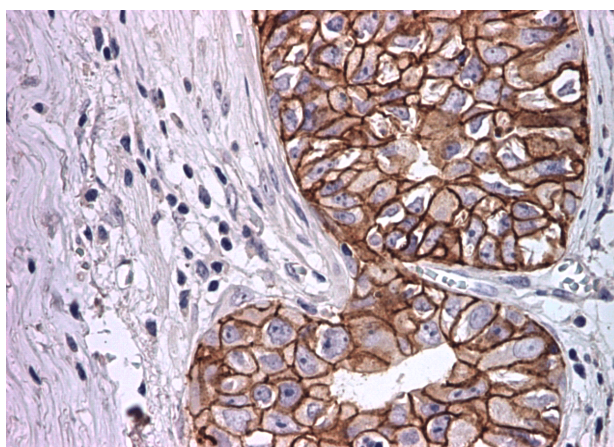


Figure 7 – Invasive mammary carcinoma: Her2 positive (3+), ER-/PR-. Anti-Her2 antibody immunostaining, ×200. Her2: Human epidermal growth factor receptor 2; ER: Estrogen receptor; PR: Progesterone receptor.

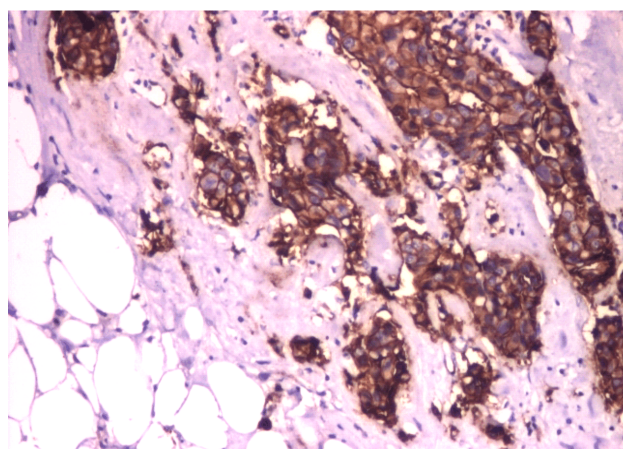


Figure 8 – Invasive mammary carcinoma: Her2 positive (3+), ER+/PR-. Anti-Her2 antibody immunostaining, ×100. Her2: Human epidermal growth factor receptor 2; ER: Estrogen receptor; PR: Progesterone receptor.

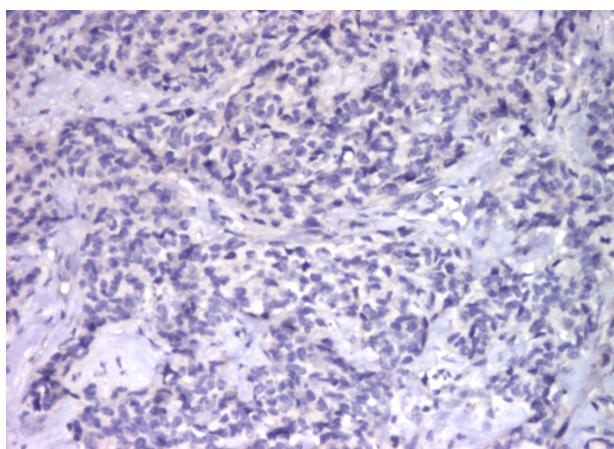


Figure 9 – Invasive mammary carcinoma: Her2 negative (0), ER-/PR-. Anti-Her2 antibody immunostaining, ×100. Her2: Human epidermal growth factor receptor 2; ER: Estrogen receptor; PR: Progesterone receptor.

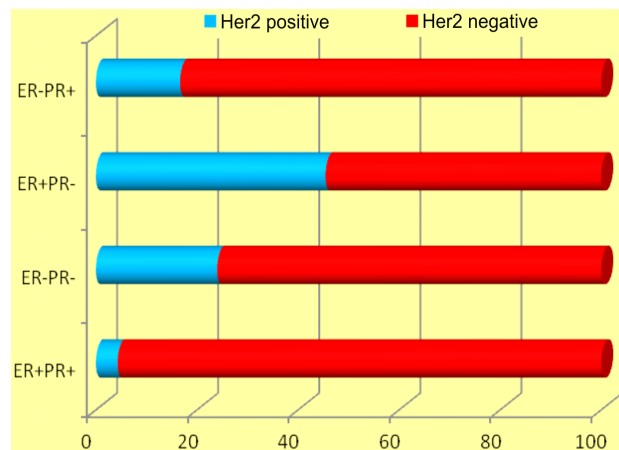


Figure 10 – The distribution of Her2 status according to hormone receptor immunophenotypes for conservative surgeries. ER: Estrogen receptor; PR: Progesterone receptor.

Table 6 – The distribution of HER2 status according to hormone receptor immunophenotypes for the groups of patients who underwent conservative surgeries

HER2 status	HER2+ (15)	HER2- (97)
ER+/PR+ (70)	3 (4.29%)	67 (95.71%)
ER-/PR- (25)	6 (24%)	19 (76%)
ER+/PR- (11)	5 (45.45%)	6 (54.55%)
ER-/PR+ (6)	1 (16.67%)	5 (83.33%)

HER2: Human epidermal growth factor receptor 2 gene; ER: Estrogen receptor; PR: Progesterone receptor.

Table 7 – The distribution of HER2 status according to hormone receptor immunophenotypes for the groups of patients who underwent radical surgeries

HER2 status	HER2+ (24)	HER2- (164)
ER+/PR+ (119)	5 (4.2%)	114 (95.78%)
ER-/PR- (41)	9 (21.95%)	32 (78.05%)
ER+/PR- (18)	8 (44.44%)	10 (55.56%)
ER-/PR+ (10)	2 (20%)	8 (80%)

HER2: Human epidermal growth factor receptor 2 gene; ER: Estrogen receptor; PR: Progesterone receptor.

Table 8 – The distribution of recurrences depending on HER2 status and the status of hormone receptors for the cases which underwent conservative surgeries

Analyzed parameters	N	No. of recurrences after conservative surgeries	Percentage
<i>Status of hormone receptors</i>			
ER+	80	9	11.25%
ER-	32	16	50%
PR+	76	9	11.84%
PR-	36	16	44.44%
<i>Hormone immunophenotypes</i>			
ER+/PR+	70	9	12.85%
ER+/PR-	11	0	0%
ER-/PR+	6	0	0%
ER-/PR-	25	16	64%
<i>HER2 status (post in situ hybridization)</i>			
HER2+	15	8	53.33%
HER2-	97	17	17.53%

HER2: Human epidermal growth factor receptor 2 gene; N: No. of cases; ER: Estrogen receptor; PR: Progesterone receptor.

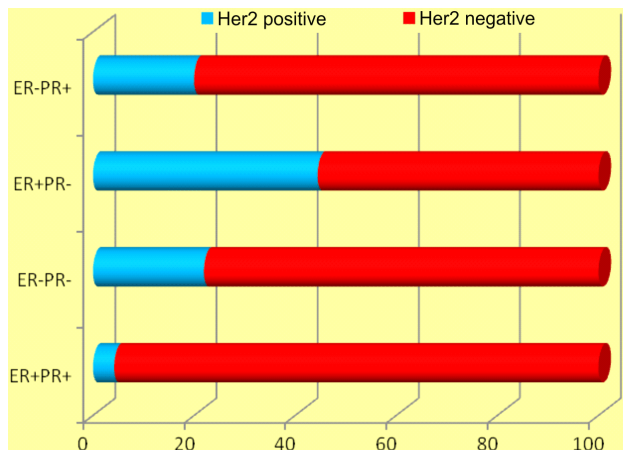


Figure 11 – The distribution of Her2 status according to hormone receptor immunophenotypes for radical surgeries. ER: Estrogen receptor; PR: Progesterone receptor.

Table 9 – The distribution of recurrences depending on HER2 status and the status of hormone receptors for the cases which underwent radical surgeries

Analyzed parameters	N	No. of recurrences after radical surgeries	Percentage
<i>Status of hormone receptors</i>			
ER+	136	7	5.15%
ER-	52	12	23.08%
PR+	129	7	5.43%
PR-	59	12	20.34%
<i>Hormone immunophenotypes</i>			
ER+/PR+	119	7	5.88%
ER+/PR-	18	0	0%
ER-/PR+	10	0	0%
ER-/PR-	41	12	29.27%
<i>HER2 status (post in situ hybridization)</i>			
HER2+	24	6	25%
HER2-	164	13	7.93%

HER2: Human epidermal growth factor receptor 2 gene; N: No. of cases; ER: Estrogen receptor; PR: Progesterone receptor.

Similarly, we noticed that the PR- mammary tumors recurred more frequently in comparison with the PR+ ones, both for the recurrences registered after the conservative surgeries and after the radical surgeries [16 (44.44%) cases vs. nine (11.84%) cases for the recurrences after the conservative surgeries; 12 (20.34%) cases vs. seven (5.43%) cases for the recurrences after the radical surgeries], the difference being also statistically significant ($p=0.039$ for the group of patients which underwent conservative surgeries and $p=0.0054$ for the group of patients which underwent radical surgeries).

As regards the hormone immunophenotypes, it was remarked that the most frequent recurrences occurred within the phenotype with both negative hormone receptors (ER-/PR-), the patients with this phenotype presenting recurrences for 16 (64%) cases after the conservative surgeries and for 12 (29.27%) cases after the radical surgeries, respectively. Next, there was the phenotype with both positive hormone receptors (ER+/PR+) out of which nine (12.85%) cases recurred after the conservative surgeries and seven (5.88%) cases recurred after the radical surgeries, respectively. The other hormone phenotypes,

the ones with one of the receptors absent, did not feature recurrences in the present study, either after the conservative or the radical surgeries. The correlations of the ipsilateral recurrences with the hormone immunophenotypes were statistically significant, the recurrences being significantly correlated with the both negative receptors phenotype ($p=0.00041$ for the group of patients who underwent a conservative surgery and $p=0.0096$ for the group of patients who underwent a radical surgery, respectively).

Analyzing the correlations between the *HER2* status (after the complete IHC analysis accompanied by CISH) and the local ipsilateral recurrences after the conservative surgeries, we noticed that these were three times more frequent in the case of *HER2*⁺ mammary carcinomas, in comparison with the *HER2*⁻ ones (53.33% vs. 17.53% of the cases). The same report was also met in the case of the radical surgeries, in what concerns the correlation between *HER2* status and the local recurrence (25% vs. 7.93% of the cases). Thus, among *HER2*⁺ tumors, eight cases recurred after the conservative surgeries and six cases recurred after the radical surgeries, while for *HER2*⁻ tumors, 17 cases recurred after the conservative surgeries and 13 cases recurred after radical surgeries. Thus, comparing the recurrences in the group of *HER2*⁺ patients with the ones in the group of *HER2*⁻ patients, we noticed statistically significant differences for both groups of patients ($p=0.024$ for the group of patients who underwent conservative surgeries and $p=0.026$ for the group of patients who underwent radical surgeries). The statistical analysis of the studied parameters in comparison with ipsilateral tumoral recurrences is represented in Table 10.

Table 10 – Statistical analysis of the studied parameters in comparison with the ipsilateral tumoral recurrences

Analyzed parameters	p conservative	p radical
<i>Status of hormone receptors</i>		
ER-/ER+	0.0078* (HS)	0.0014* (HS)
PR-/PR+	0.0029* (HS)	0.0054* (HS)
<i>HER2 status (post in situ hybridization)</i>		
<i>HER2</i> ⁻ / <i>HER2</i> ⁺	0.024* (S)	0.026* (S)

ER: Estrogen receptor; PR: Progesterone receptor; *HER2*: Human epidermal growth factor receptor 2 gene; HS: Highly significant; S: Significant; * $p<0.05$.

Discussions

From the total of 1358 cases of infiltrating mammary/breast carcinomas operated in the period between 1995–2012 (502 cases which underwent conservative surgeries and 856 cases which underwent radical surgeries), a number of 300 cases of infiltrating mammary/breast carcinomas was selected for the IHC study (112 cases which underwent conservative surgeries and 188 which underwent radical surgeries). This IHC study followed establishing the ER and PR immunoexpression, as well as of the expression of the Her2/neu oncoprotein. We mention that in the IHC study there were included all the cases that recurred locally, including the 25 cases of infiltrating mammary/breast carcinomas which underwent conservative surgeries and presented local recurrences, as well as the 19 cases of infiltrating carcinomas which

underwent radical surgeries and presented local recurrences.

In our study, we firstly analyzed the nuclear immunomarking for the hormones (ER and PR) and the membrane marker for Her2/neu oncoprotein. Subsequently, the analyzed carcinomas were divided into four groups, depending on the hormones phenotypes: ER+/PR+, ER+/PR-, ER-/PR- and ER-/PR+.

The analysis of the ER immunostaining highlighted the presence of the ERs in 80 (71.42%) cases of the ones which underwent conservative surgeries and in 136 (72.34%) cases from the 188 which underwent radical surgeries. Similarly, the presence of PR immunomarker was noticed for 76 (67.86%) cases which underwent conservative surgeries and for 129 (68.61%) cases with a radical mastectomy. The statistical analysis did not reveal significant differences concerning the positivity rate of ER and PR in the group of patients who underwent conservative surgeries, in comparison with the group which underwent radical surgeries ($p=0.864$ and $p=0.89$, respectively).

The positivity rate of the two types of hormone receptors in our study was also reported by Rouanet *et al.* (2014) in the study made on the patients who underwent conservative surgeries or radical surgeries for mammary/breast tumors of small dimensions (T1a and T1b), where they observed that most of the patients (91%) present tumors with positive hormone receptors (ER or PR), 87% of the cases being ER+ tumors [16].

Subsequently, each of the cases which underwent conservative and radical surgeries were classified depending on one of the following four immunophenotypes: ER+/PR+ classical phenotype (70 cases which underwent conservative surgeries and 119 cases which underwent radical surgeries); ER-/PR- phenotype (25 cases which underwent conservative surgeries and 41 cases which underwent radical surgeries); ER+/PR- phenotype (11 cases which underwent conservative surgeries and 18 cases which underwent radical surgeries); and ER-/PR+ phenotype (six cases which underwent conservative surgeries and 10 cases which underwent radical surgeries). The most frequently met hormone immunophenotype was the classical one, with both positive hormone receptors (ER+/PR+), both for the cases which underwent conservative surgeries (70 cases, 63.416%), as well as in the case of patients with radical mastectomy (119 cases, 63.3%). For the immunophenotype with both negative hormone receptors (ER-/PR-), there were 25 (21.95%) cases which underwent conservative surgeries and 41 (21.81%) cases which underwent radical surgeries. The immunophenotypes with one positive hormone receptor (ER+/PR- and ER-/PR+, respectively) included the fewest cases, being represented by 11 (9.82%) and six (5.36%) cases, respectively, for conservative surgeries, and by 18 (9.57%) and 10 (5.32%) cases, respectively, for radical surgeries.

The results were similar to those of other researchers, who noticed that approximately 50% of the invasive mammary carcinomas express both types of hormone receptors, and approximately 25% lack in ER and PR [17]. In what concerns the cases with a heterogeneous phenotype, it seems that the benefit of hormone therapy decreases to below half the cases in which one of the receptors is missing, in comparison with the cases which

express both types of receptors. Thus, studies show that the ER+/PR- phenotype represent a distinct subgroup of mammary/breast carcinomas, as they present aggressive clinical and biological characteristics and have the most diminished benefit with hormone therapy, in comparison with all the other phenotypes [18].

The analysis of Her2 oncoprotein immunomarker allowed classifying the 112 mammary carcinomas which underwent conservative surgeries, depending on Her2 score, thus: score 0 – 46 (41.08%) cases, score 1+ – 38 (33.92%) cases, score 2+ – 22 (19.64%) cases, and score 3+ – six (5.36%) cases. The cases who underwent radical surgeries were classified depending on Her2 score: score 0 – 77 (40.96%) cases, score 1+ – 64 (34.04%) cases, score 2+ – 38 (20.21%) cases, and score 3+ – nine (4.79%) cases. Following the CISH examination in the group of patients who underwent conservative surgeries, there were 15 cases with *HER2*+ status, six cases with IHC 3+ score, and nine cases with IHC 2+ score, which presented the amplification of *HER2* gene after *in situ* hybridization. In the group of patients who underwent radical surgeries, there were 24 cases with *HER2*+ positive, nine cases with IHC 3+ score and 15 cases with IHC 2+ score, and the amplification of the *HER2* gene after *in situ* hybridization. Thus, the group which underwent a conservative surgery included 15 (13.39%) *HER2*+ cases and 97 (86.61%) *HER2*- cases, and the group which underwent a radical surgery included 24 (12.76%) *HER2*+ cases and 164 (87.24%) *HER2*- cases. According to the statistics, 20–30% of the mammary carcinomas present *HER2* receptor overexpression and it is associated with an unfavorable prognosis, being a predictive risk factor for survival [19].

Following the correlations between the immunohistochemically-studied parameters (hormone receptors and Her2 oncoprotein), we observed that Her2 positivity is associated more frequently with the ER+/PR- phenotype (45.45% of the cases which underwent conservative surgeries and 44.44% of the cases which underwent radical surgeries), this association being statistically significant both for the cases which underwent conservative surgeries ($p=0.0085$), as well as for the cases which underwent radical surgeries ($p=0.00028$). The absence of Her2 oncoprotein was associated more frequently with the ER+/PR+ phenotype (95.71% of the cases which underwent conservative surgeries and 95.78% of the cases which underwent radical surgeries). Between the *HER2*-status and the hormone phenotypes there are not statistically significant correlations ($p=0.758$ for the cases which underwent conservative surgeries and $p=0.552$ for the cases which underwent radical surgeries). In the study of Rouanet *et al.* [16], when they took into consideration the groups depending on *HER2* status and the status of hormone receptors (HR), the distribution of the patients was the following: *HER2*-/HR+ in 87% of the cases; *HER2*-/HR- in 7% of the cases; *HER2*+/HR+ in 4% of the cases; and *HER2*+/HR- in 2% of cases, the results being similar to the ones obtained in our study. The same study shows that *HER2*+/HR+ tumors have the best prognosis, with a survival global rate of 94% and survival without disease of 89%, at 10 years. Moreover, in the study of Arvold *et al.*, referring to local recurrences after the breast conservative surgery, *HER2*- and HR+

cases represented the great majority (mammary cancer of luminal A and luminal B types represented 76.91% of the cases) [20].

In what concerns the IHC analyzed ipsilateral local recurrences, in the case of invasive mammary carcinomas which underwent conservative and radical surgeries, respectively, we observed that these recurrences were much more frequent for ER- tumors in comparison with ER+ tumors (50% vs. 11.25% for recurrences after conservative surgeries and 23.08% vs. 5.15% for recurrences after radical surgeries). The statistical analysis proved that recurrences were more frequent statistically significant for ER- patients in comparison with the ones who had ER+ primary tumor ($p=0.0078$ for the patients who underwent conservative surgeries and $p=0.0014$ for the ones who underwent radical surgeries). Similarly, we observed that PR- mammary tumors recurred more frequently in comparison with PR+ ones, both for recurrences registered after conservative surgeries and after radical surgeries (44.44% vs. 11.84% for recurrences after conservative surgeries and 34% vs. 5.43% for recurrences after radical surgeries), the differences being, also, statistically significant ($p=0.039$ for the group of patients who underwent conservative surgeries and $p=0.0054$ for the group of patients who underwent radical surgeries).

The study of Voduc *et al.* [3], done on 2985 mammary tumors which underwent either conservative or radical surgeries, showed that local and regional recurrences at patients with luminal A-type tumors (ER+/PR+/Her2-), which underwent conservative surgeries, had a rate of only 8% and 3%, respectively, 10 years after the surgery and the prognosis was the most favorable. Similarly, the same study shows that in the group with a mastectomy, the univariate analysis exhibited statistically significant differences about locoregional recurrences. Thus, after mastectomy, the patients with luminal A-type tumors had the best prognosis and the lowest rates of local and regional recurrences 10 years after the surgery (8% and 4%, respectively) [3].

In what concerns hormones immunophenotypes, we noticed that the most frequent recurrences appeared in the case of the phenotype with both negative hormone receptors (ER-/PR-), the patients with this phenotype presenting recurrences in 16 (64%) cases after conservative surgeries and in 12 (29.27%) cases after radical surgeries. Next was the both positive receptors phenotype (ER+/PR+) of which nine (12.85%) cases recurred after the conservative surgeries and seven (5.88%) cases after radical surgeries. The other hormone phenotypes, such as the ones with one negative receptor, did not present any recurrences in our study either after conservative surgeries or radical surgeries. The correlations between the ipsilateral recurrences and the hormones immunophenotypes were statistically significant, the recurrences correlating with the phenotype with both negative receptors no matter the type of the surgeries ($p=0.00041$ for the group of patients who underwent a conservative surgery and $p=0.0096$ for the group of patients who underwent a radical surgery).

Analyzing the correlations between Her2 status (after the complete IHC analysis along with the CISH) and the local ipsilateral recurrences after the conservative surgeries, we noticed that they were three times more frequent in

the case of Her2+ mammary carcinomas in comparison with the Her2- ones (53.33% vs. 17.53% of the cases). The same report was also met in the case of radical surgeries concerning the correlation between the Her2 status and the local recurrence (25% vs. 7.93% of the cases). Thus, of the Her2+ tumors, eight cases recurred after conservative surgeries and six cases after radical surgeries, while from Her2- tumors, 17 cases recurred after conservative surgeries and 13 cases after radical surgeries. Thus, comparing the recurrences occurred in the group of Her2+ patients with the ones which occurred in the group of Her2- patients, we obtained statistically significant differences, the Her2+ tumors presenting recurrences much more frequently, no matter the type of the surgery ($p=0.024$ for the group of the patients who underwent conservative surgeries and $p=0.026$ for the group of the patients who underwent radical surgeries).

The analysis performed by Ishitobi *et al.* (2012) [21] supports the results of the present work, the authors observing that none of the patients who underwent conservative surgeries and who had an ER+ and Her2- mammary carcinoma did not develop tumoral ipsilateral recurrences and the frequency of recurrences for this subgroup was significantly lower than for the patients with another status of ER and Her2. For the patients who underwent radical surgery (mastectomy), a Danish trial on 1000 patients, analyzing the prognosis and predictive values of ER, PR and Her2, showed that the triple negative sub-type of mammary tumors (ER-/PR-/Her2-) and the Her2+ subtype (ER-/PR-/Her2+) represent predictive independent factors for locoregional recurrences [22]. Moreover, our results were in accordance with a series of other results of previous studies which show that luminal A mammary tumors (with both HR+ and Her2-) have the best prognosis in what survival is concerned [23].

In the case of patients with a mastectomy, in the univariate analysis, the ER+ or PR+ cases, or the combination of ER+ with PR+, were strongly associated with a reduced rate of locoregional recurrences, while the basal mammary carcinoma (ER-/PR-/Her2-, triple negative) is associated with a high rate of locoregional recurrences. Consequently, this triple negative phenotype must be considered together with other predictive factors of locoregional recurrences when patients are selected for post-mastectomy radiotherapy [23, 24].

Our study proves that IHC markers, represented by ERs and PRs, along with Her2 oncoprotein (markers with major implications in the therapy of mammary cancer) can be very useful for the identification of patients with a high of local recurrences after conservative and radical surgery of mammary cancer. Still, the biological mechanisms, which are at the basis of these observations, remain too little known [25–28].

✉ Conclusions

The total resection of the sternum is feasible in selected cases of oligometastatic breast cancer involving only the sternum. Mammary carcinomas express ER and PR in the majority of cases, without any significant differences regarding the positivity rate of ER and PR, in the cases of the patients who underwent conservative surgeries, in comparison with the ones who underwent radical surgeries.

The local recurrences are more frequent statistically significant in the case of ER- patients, in comparison with patients having ER+ primary tumor, no matter the type of the surgery. The IHC markers represented by ER and PR, and by the Her2 oncoprotein, can be very useful for the identification of the patients who present a high risk of local recurrences after conservative or radical surgery of mammary cancer.

Conflict of interests

The authors declare that they have no conflict of interests.

Authors' contributions

Tiberiu Ștefăniță Țenea-Cojan and Adrian Macovei contributed equally to this paper and share first authorship.

References

- [1] Lucamba Ituamba EDG, Lv JX, Bounda GA, Cao XH, Hu HL, Zhang YN. The progressive journey from ductal carcinoma *in-situ* into invasive breast cancer: an extensive systematic literature review on biomarkers. *Clin Oncol Case Rep*, 2018, 1:2.
- [2] Rakha EA, Reis-Filho JS, Baehner F, Dabbs DJ, Decker T, Eusebi V, Fox SB, Ichihara S, Jacquemier J, Lakhani SR, Palacios J, Richardson AL, Schnitt SJ, Schmitt FC, Tan PH, Tse GM, Badve S, Ellis IO. Breast cancer prognostic classification in the molecular era: the role of histological grade. *Breast Cancer Res*, 2010, 12(4):207.
- [3] Voduc KD, Cheang MC, Tyldesley S, Gelmon K, Nielsen TO, Kennecke H. Breast cancer subtypes and the risk of local and regional relapse. *J Clin Oncol*, 2010, 28(10):1684–1691.
- [4] Ariga R, Zarif A, Korasick J, Reddy V, Siziopikou K, Gattuso P. Correlation of Her-2/neu gene amplification with other prognostic and predictive factors in female breast carcinoma. *Breast J*, 2005, 11(4):278–280.
- [5] Garcia S, Dalès JP, Charafe-Jauffret E, Carpentier-Meunier S, Andrac-Meyer L, Jacquemier J, Andonian C, Lavaut MN, Allasia C, Bonnier P, Charpin C. Poor prognosis in breast carcinomas correlates with increased expression of targetable CD146 and c-Met and with proteomic basal-like phenotype. *Hum Pathol*, 2007, 38(6):830–841.
- [6] Ciocca DR, Gago FE, Fanelli MA, Calderwood SK. Co-expression of steroid receptors (estrogen receptor alpha and/or progesterone receptors) and Her-2/neu: clinical implications. *J Steroid Biochem Mol Biol*, 2006, 102(1–5):32–40.
- [7] Ayadi L, Khabir A, Amouri H, Karay S, Dammak A, Guermazi M, Boudawara T. Correlation of HER-2 over-expression with clinico-pathological parameters in Tunisian breast carcinoma. *World J Surg Oncol*, 2008, 6:112.
- [8] Zhou B, Yang DQ, Xie F. Biological markers as predictive factors of response to neoadjuvant taxanes and anthracycline chemotherapy in breast carcinoma. *Chin Med J (Engl)*, 2008, 121(5):387–391.
- [9] Bang YJ, Van Cutsem E, Feyereislova A, Chung HC, Shen L, Sawaki A, Lordick F, Ohtsu A, Omuro Y, Satoh T, Aprile G, Kulikov E, Hill J, Lehle M, Rüschoff J, Kang YK, ToGA Trial Investigators. Trastuzumab in combination with chemotherapy *versus* chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase 3, open-label, randomised controlled trial. *Lancet*, 2010, 376(9742):687–697.
- [10] Arnaud da Cruz P, Lopes C. Implications of different cancer stem cell phenotypes in breast cancer. *Anticancer Res*, 2017, 37(5):2173–2183.
- [11] Haffty BG, Yang Q, Reiss M, Kearney T, Higgins SA, Weidhaas J, Harris L, Hait W, Toppmeyer D. Locoregional relapse and distant metastasis in conservatively managed triple negative early-stage breast cancer. *J Clin Oncol*, 2006, 24(36):5652–5657.
- [12] Nguyen PL, Taghian AG, Katz MS, Niemierko A, Abi Raad RF, Boon WL, Bellon JR, Wong JS, Smith BL, Harris JR. Breast cancer subtype approximated by estrogen receptor, progesterone receptor, and HER-2 is associated with local and distant recurrence after breast-conserving therapy. *J Clin Oncol*, 2008, 26(14):2373–2378.

- [13] Kyndi M, Sørensen FB, Knudsen H, Overgaard M, Nielsen HM, Overgaard J; Danish Breast Cancer Cooperative Group. Estrogen receptor, progesterone receptor, HER-2, and response to postmastectomy radiotherapy in high-risk breast cancer: the Danish Breast Cancer Cooperative Group. *J Clin Oncol*, 2008, 26(9):1419–1426.
- [14] Wolff AC, Hammond ME, Schwartz JN, Hagerty KL, Allred DC, Cote RJ, Dowsett M, Fitzgibbons PL, Hanna WM, Langer A, McShane LM, Paik S, Pegram MD, Perez EA, Press MF, Rhodes A, Sturgeon C, Taube SE, Tubbs R, Vance GH, van de Vijver M, Wheeler TM, Hayes DF; American Society of Clinical Oncology/College of American Pathologists. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. *Arch Pathol Lab Med*, 2007, 131(1):18–43.
- [15] Allred DC, Harvey JM, Berardo M, Clark GM. Prognostic and predictive factors in breast cancer by immunohistochemical analysis. *Mod Pathol*, 1998, 11(2):155–168.
- [16] Rouanet P, Roger P, Rousseau E, Thibault S, Romieu G, Mathieu A, Cretin J, Barneon G, Granier M, Maran-Gonzalez A, Daures JP, Boissiere F, Bibeau F. HER2 overexpression a major risk factor for recurrence in pT1a-bN0M0 breast cancer: results from a French regional cohort. *Cancer Med*, 2014, 3(1):134–142.
- [17] Bardou VJ, Arpino G, Elledge RM, Osborne CK, Clark GM. Progesterone receptor status significantly improves outcome prediction over estrogen receptor status alone for adjuvant endocrine therapy in two large breast cancer databases. *J Clin Oncol*, 2003, 21(10):1973–1979.
- [18] Arpino G, Weiss H, Lee AV, Schiff R, De Placido S, Osborne CK, Elledge RM. Estrogen receptor-positive, progesterone receptor-negative breast cancer: association with growth factor receptor expression and tamoxifen resistance. *J Natl Cancer Inst*, 2005, 97(17):1254–1261.
- [19] Engel RH, Kaklamani VG. HER2-positive breast cancer: current and future treatment strategies. *Drugs*, 2007, 67(9):1329–1341.
- [20] Arvold ND, Taghian AG, Niemierko A, Abi Raad RF, Sreedhara M, Nguyen PL, Bellon JR, Wong JS, Smith BL, Harris JR. Age, breast cancer subtype approximation, and local recurrence after breast-conserving therapy. *J Clin Oncol*, 2011, 29(29):3885–3891.
- [21] Ishitobi M, Ohsumi S, Inaji H, Ohno S, Shigematsu H, Akiyama F, Iwase T, Akashi-Tanaka S, Sato N, Takahashi K, Oura S. Ipsilateral breast tumor recurrence (IBTR) in patients with operable breast cancer who undergo breast-conserving treatment after receiving neoadjuvant chemotherapy: risk factors of IBTR and validation of the MD Anderson Prognostic Index. *Cancer*, 2012, 118(18):4385–4393.
- [22] Overgaard M, Jensen MB, Overgaard J, Hansen PS, Rose C, Andersson M, Kamby C, Kjaer M, Gadeberg CC, Rasmussen BB, Blichert-Toft M, Mouridsen HT. Postoperative radiotherapy in high-risk postmenopausal breast-cancer patients given adjuvant tamoxifen: Danish Breast Cancer Cooperative Group DBCG 82c randomised trial. *Lancet*, 1999, 353(9165):1641–1648.
- [23] Sørli T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, van de Rijn M, Jeffrey SS, Thorsen T, Quist H, Matese JC, Brown PO, Botstein D, Lønning PE, Børresen-Dale AL. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci U S A*, 2001, 98(19):10869–10874.
- [24] Eremia IA, Ciobanu M, Tenea T, Comănescu MV, Crăitoiu Ș. Invasive papillary carcinoma of the mammary gland: histopathologic and immunohistochemical aspects. *Rom J Morphol Embryol*, 2012, 53(3 Suppl):811–815.
- [25] Bădescu A, Georgescu CV, Vere CC, Crăitoiu Ș, Grigore D. Correlations between Her2 oncoprotein, VEGF expression, MVD and clinicopathological parameters in gastric cancer. *Rom J Morphol Embryol*, 2012, 53(4):997–1005.
- [26] Khan AJ, Milgrom SA, Barnard N, Higgins SA, Moran M, Shahzad H, Kim S, Goyal S, Al-Faraj F, Kirstein L, Kearney T, Haffty BG. Basal subtype, as approximated by triple-negative phenotype, is associated with locoregional recurrence in a case-control study of women with 0–3 positive lymph nodes after mastectomy. *Ann Surg Oncol*, 2014, 21(6):1963–1968.
- [27] Burada F, Dumitrescu T, Nicolai R, Ciurea ME, Rogoveanu I, Ioana M. Cytokine promoter polymorphisms and risk of colorectal cancer. *Clin Lab*, 2013, 59(7–8):773–779.
- [28] Nuyten DS, Kreike B, Hart AA, Chi JT, Sneddon JB, Wessels LF, Peterse HJ, Bartelink H, Brown PO, Chang HY, van de Vijver MJ. Predicting a local recurrence after breast-conserving therapy by gene expression profiling. *Breast Cancer Res*, 2006, 8(5):R62.

Corresponding authors

Andrei Ionuț Costin, MD, PhD Student, Department of Surgery, University of Medicine and Pharmacy of Craiova, Railroads Hospital of Craiova, 6 Știrbei Vodă Avenue, 200374 Craiova, Dolj County, Romania; Phone +40251–532 436, e-mail: andrei_costin94@yahoo.com

Adrian Macovei, Teaching Assistant, MD, PhD, Department of Extreme Conditions Medicine, University of Medicine and Pharmacy of Craiova, 2 Petru Rareș Street, 200349 Craiova, Dolj County, Romania; Phone +40351–443 561, e-mail: adrian.macovei@gmail.com

Received: January 16, 2018

Accepted: November 15, 2018