Immunohistochemical Analisys in Breast Carcinoma Treated with Conservative Surgery

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Breast cancer is the most common type of cancer in women worldwide and the second most common cancer overall, making it a major international concern from both socio and economical point of view. World Health Organisation (WHO) estimated that worldwide over 508.000 women died in 2011 due to breast cancer. American Cancer Society's (ACS) study conducted in 2017 showed important numbers about the burden of breast cancer. Therefore, they estimated a number of 252,710 new breast cancer to be diagnosed in women and 2,470 in men in 2017- only in America. On 1st of January , there were about 3.5million US women with a history of breast cancer. The paper presents the correlations between recurrences of the tumor in breast cancer trated with conservative surgery and their immunohistochemical expression.

Keywords: conservative surgery, immunohistochemistry, infiltrative breast carcinoma, tumour reoccurrences

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Experimental part
Aim of the study
The aim of the presented study was to find correlations between recurrences of the tumour in breast cancer treated with conservative surgery and their immunohystochemical expression .

Material and method
The study material was formed of breast tissue samples taken from 100 operated patients for invasive breast carcinoma, using conservative breast surgery, in the Surgical department at The University CF Hospital Craiova, during 15 years, between 2003 and 2018. A total of 21 cases out of the 100 were carcinomas that reoccurred following the initial conservative procedure.

The immunohystochemical analyses employed was one based on soluble complexes of immuno-enzymes, called LSAB/HRP (labelled streptavidin biotin). In the immunohystochemical analyses we used antibodies produced by DAKO Cytomation, Denmark.

In the table below (table 1) we present the dilutions and pre-treatment of these antibodies CISH and HER2 techniques.

From the blocks of paraffin analysed though IHC for HER2 that received an ambivalent 2+ score, we extracted sections 4-5 um thick that were exposed on Superfrost/ plus plaque and these sections were subject to in situ chromogen hybridization exam (CISH).

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Antibody	Clone	Dilution	Pretreatment	Incubation time	External control
ER	1D5	1:50	EDTA ph=8, 20 min MW	1 h at 37⁰C	Breast carcinoma
PR	PgR636	1:50	EDTA ph=8, 20 min MW	1 h at 37⁰C	Breast carcinoma
Her 2	policlonal	1:250	Citrat ph = 6, 20 min MW	30 min at room temperature	Breast carcinoma

Table 1
ANTIBODIES USED, CLONE, DILUTION AND TREATMENT

For the interpretation of the results for the Her 2 markings we used the ASCO and CAP recommended criteria, 2007 (Wolff, A, Hammond, E, et al 2007).

To quantify the status of the hormonal receptors we used the Allred score (Allred C ºi col., 1999). Tumours with an Allred score d" 2 were considered negative and those with and Allred score > 2 were thought of as positive.

The cases that were evaluated though the CISH method was analysed as follows:

-Amplification (high or low) - result was CISH Her 2 positive. Tumours 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 tumour cells for the area chosen as significant for analysing. The tumour 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 tumour cells analysed.

-Lack of amplification -result was CISH Her 2 negative - these were either tumours with polysomy of the chromosome 17 : presenting with 3-5 points/nuclei in more than 50% of all the tumour cells from the selected area; or diploid tumours with normal status for the HER2 gene with 1-2 pints/nuclei in more than 50% of all tumour cells from the selected area.

Results and discussions

The histology of the 100 studied cases, using haematoxylin - eosin staining, allowed there grouping, according to WHO criteria in: invasive ductal carcinomas NOS - 60 cases, invasive lobular carcinomas - 25 cases, squamous carcinomas - 10 cases and tubular carcinomas - 5 cases.

The degree of differentiation for tumours (the degree of histological malignancy) evaluated according to the Nottingham scale allowed the tumours to be separated into: G1 invasive mammary carcinomas -8 cases, G2 invasive mammary carcinomas -50 cases and G3 invasive mammary carcinomas -42 cases.

According to the pTNM classification, at the time of diagnosis, we included tumours that were divided as follows: Stage I -10 cases, Stage IIA -44 cases, Stage IIB - 18 cases, stage IIIA – 20 cases and stage IIIB -8 cases.

The analyses of the immunostaining for hormonal receptors (ER and PR), allowed the separation of all tumours into 4 lots (immune-phenotypes) that we eventually correlated with the Her 2 status and the clinical and morphological characteristics, some with known prognostic values. The cases that had ipsilateral reoccurrences of the malignancy were analysed separately in respect to the expression of the hormonal receptors and the her 2 status.

The analyses of immunostaining for ER showed the presence of oestrogen receptors in 66 cases (66%), while the rest of 34 cases (34 %) were negative for ER. Similarly,

58 of the cases were PR positive (58%) and 42 of the cases were PR negative (42%).

Eventually, each of the 100 immunohistochemically analysed cases, were assigned to one of four phenotypes: classical phenotype ER+/PR+ (60 cases), ER-/PR- phenotype (24 cases), ER+/PR-phenotype (11 cases) and ER-/PR+ phenotype (5 cases) (fig. 1).

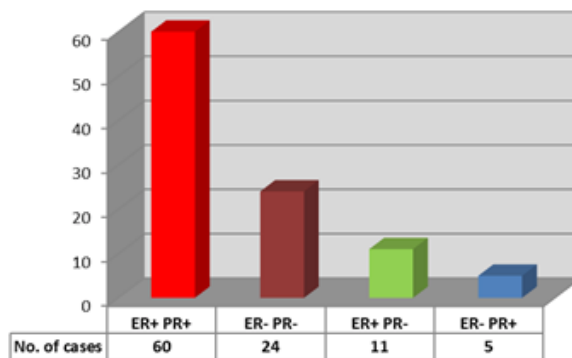|	ER+ PR+	ER- PR-	ER+ PR-	ER- PR+
No. of cases	60	24	11	5

Fig. 1 Distribution of cases based on the immune-phenotype of hormonal receptors

The most commonly seen hormonal immunophenotype was the classical one with both receptors being present ER+/PR+. The interpretation of immunostaining of Her2 oncoprotein was done according ASCO-CAP recommendations, cases being classed as: score 0: 44 cases (44%); score 1+ :30 cases (30%); score 2+: 20 cases (20%) and score 3+: 6 cases (6%).

The assignment of the analysed cases according to Her 2 status was complete following in situ hybridization and showed 12 Her 2 positive cases (12%) and 88 Her2 negative cases (88%).
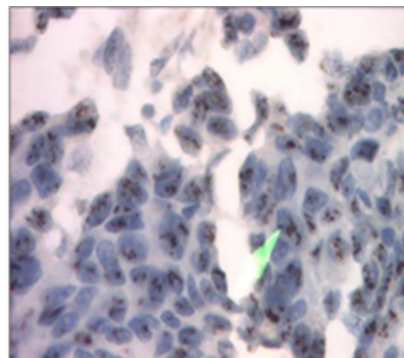

Fig. 2 Amplification of the Her2 gene, Her2 CISH positive X 20

In the next stage we monitored for the correlations between hormonal receptors and the status of the Her2/ neu oncoprotein, assigned according to ASCO-CAP immunohistochemical score (table 2).

We observed that Her 2 negative tumours (with 0 and 1+ scores) were lacking ER receptors in 24 out of 74 cases, meaning 32.43% of cases, while her2 2+ and 3+ were lacking ER receptors in 6 out of 26 cases, meaning 23.07% of the cases, the difference being statistically not significant ($p=0.568$). Regarding the PR receptors, we noticed that

Hormonal immune-phenotype	Score 0 N=44	Score 1+ N=30	Score 2+ N=20	Score 3+ N=6
ER+PR+	23	28	9	0
ER-PR-	18	0	0	6
ER+PR-	0	0	11	0
ER-PR+	3	2	0	0

Table 2
THE DISTRIBUTION OF THE HER2/NEU SCORE BASED ON ER AND PR IMMUNO-PHENOTYPE

Her2 negative tumours were lacking in progesterone receptors in 18 out of 74 cases, meaning 24.32% of cases, while Her 2 2+ and 3+ tumours were lacking in PR receptors in 17 out of 26 cases, meaning 65.38% of the cases, the difference being statistically significant (p=0.045). Her2 negative tumours lacking in both hormonal receptors (triple negative tumours or *basal-like*, representing a particular group of malignancies) were found in 22 out of the 100 studied cases though IHC, meaning a percentage of 22% of the cases.

The CISH testing allowed carcinomas were divided into Her 2 positive or Her2 negative based on hormonal phenotypes. Relating to hormonal phenotypes we noticed tumours that had both receptors positive (phenotype ER+PR+) and more frequently Her2 negative then hher2 positive (97.21% vs 2.79%). On the other hand tumours with both hormonal receptors absent (ER-PR-phenotype) were Her2 negative in 86.33% of all cases and Her positive in 13.67% of the cases.

Mammary carcinomas that were only lacking in PR receptors (ER+PR- phenotype) were equally Her2 positive and Her2 negative (50% *vs* 50%) while carcinomas lacking in ER receptors (ER-PR+) were Her2 negative in 100% of the cases. Therefore we noticed that Her2 positivity was more likely to be associated with ER+PR- phenotype (50% of the cases) while the absence of Her2 was more often associated with ER+PR+ (97.21%) phenotype. We noticed highly significantly statistic relationships between the Her2 status and the hormonal phenotype (=0.00068).

Last but not least, we analyzed the immuno-hystochemical reoccurrences and we found the results highlighted in the table 3.

As we can see in the table above the ipsilateral local reoccurrences, for the invasive breast carcinomas that were analysed though IHC, were often associated with negative tumours for oestrogen receptors as opposed to tumours with positive ER receptors (9 cases vs 6 cases, meaning 26.47% of the cases vs 9.09%). Statistical analyses
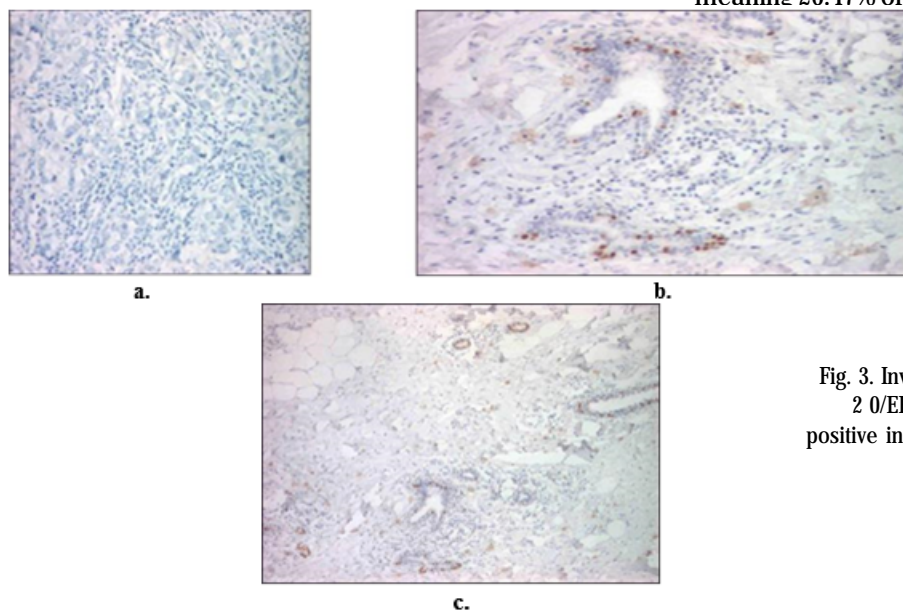

Fig. 3. Invasive mammary carcinoma (IE patient) her 2 0/ER-/PR-; (a.Her2 0 negative, X 100; b. ER -, positive internal control, x 100; c. PR-, positive internal control, x 40)
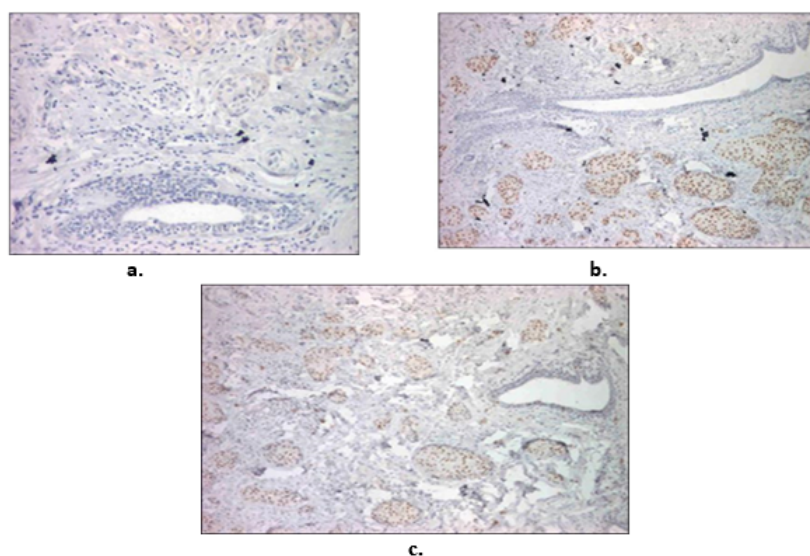

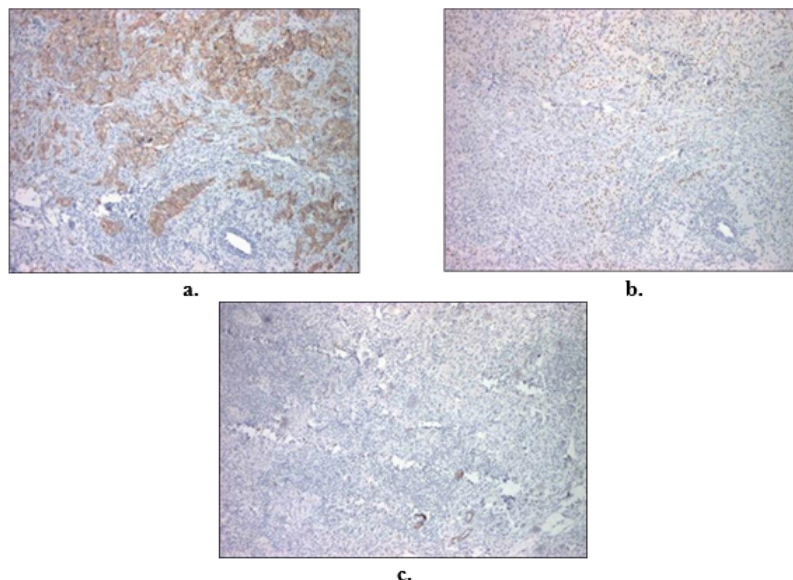Fig. 4. Invasive mammary carcinoma (patient SE) Her2 1+/ER+/PR=(a. Her2 1+ negative, X 100; b. ER +, x 100, c. PR+, x40)

Fig. 5. Invasive mammary carcinoma (patient ZG) Her2 2+/ER+/PR- (a.Her2 2+ ambivalent, x 100; b. ER+, x 40; c.PR -, positive internal control x 40)
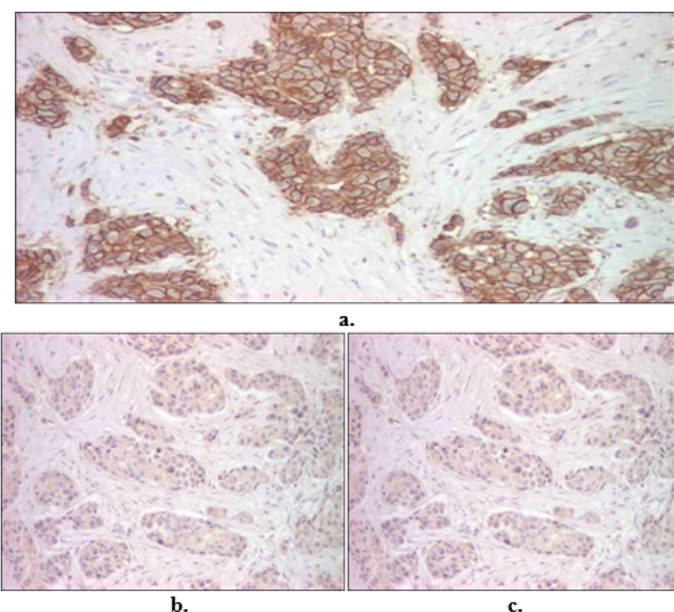

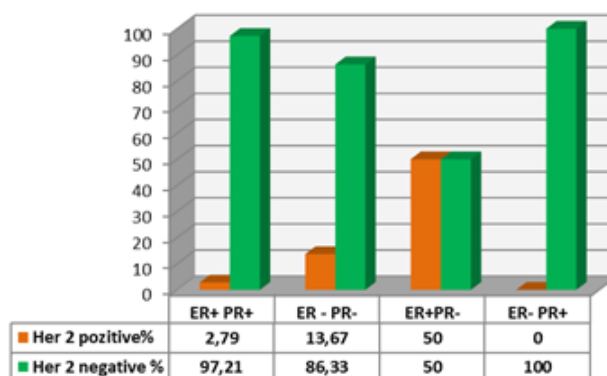Fig. 6 Invasive mammary carcinoma (patient CS) Her2 3+/ER-/PR- (a.Her2 3+ positive, x100; b. ER-, x100; c. PR-, x100)

Fig. 7. Distribution of the Her2 status based on immune-phenotypes for hormonal receptors

Analysed parameters	n	Number of reocurrences	%
Status of hormonal receptors			
ER +	66	6	9.09%
ER -	34	9	26.47%
PR +	58	6	10.34%
PR -	42	9	21.42%
Hormonal immune-phenotypes			
ER +/PR +	60	6	10%
ER +/PR -	11	0	0%
ER -/PR +	5	0	0%
ER -/PR -	24	9	37.5%
HER2 status (post in situ hybridisation)			
HER2 +	12	4	33.33%
HER2 -	88	10	11.36%

Table 3
DISTRIBUTION OF REOCCURRENCES BASED ON HER 2 STATUS AND HORMONAL RECEPTOR STATUS

showed that reoccurrences were statistically significant more frequent in patient with absent oestrogen receptors compared to those with present oestrogen receptors (p=0.022). Similarly we noticed that breast cancer with absent progesterone receptors were more likely to reoccur compared to those with present progesterone receptors (9 cases vs 6 cases, meaning 21.42% of cases compared to 10.34%), the difference being of statistical significance as well (p=0.043).

In the case of hormonal immuno-phenotypes we noticed that the most frequent reoccurrences had both receptors absent (ER-/PR-), a total of 9 cases, representing 37.5% of all cases. This was followed by the phenotype that had both receptors positive (ER+/PR+) with 6 reoccurrences,

representing 10% of cases. Other hormonal phenotypes, with one hormonal receptor absent had no reoccurrence in our study. The correlation between ipsilateral reoccurrence of breast carcinoma and hormonal immune-phenotype were statistically significant (p=0.033).

Analysing the correlations between the Her2 status (after complete IHC analyses followed by CISH) and ipsilateral local reoccurrences we noticed that they were more likely to happen for Her2 positive mammary carcinomas compared to her2 negative carcinomas (33.33% of cases vs 11,36% of cases). Therefore from the Her2 positive tumours we had 10 reoccurrences, while from the Her2 negative group we had 4 reoccurrences. Despite these results when comparing the two lots statistically there were no significant differences (p=0.288).

In this particular study the analyses of the immunostaining for ER showed the presence of oestrogen receptors in 66 of the 100 cases analysed (66%), the 34 remaining cases (34%) being negative for ER. Analysing the presence of immune-marking for progesterone receptors we had results similar to those for ER receptors. A total of 58 cases were PR positive (58%) and 42 cases were PR negative (42%). Statistical analyses did not reveal any significance between the degree of positivity for the 2 markers (p=0.521). The degree of positivity for the 2 types of hormonal receptors (66% vs 58%) is similar to rate measured in all types of breast cancer no matter the type of surgical intervention and similar to the results observed in studies done of larger cohorts of patients [1-4] although variations may sometimes be more evident reported to the degree of positivity for the 2 receptors [5-7].

Following the separation of the cases studied in 4 hormonal phenotypes we observed that the most frequently encountered phenotype was the classical one with both receptors positive ER+/PR+ which was recorded for a number of 60 cases (60%). For the immune-phenotype with both receptors negative, ER-/PR- we had 24 cases (24%). The results were comparable to those obtained in similar studies that concluded that almost 50% of invasive mammary carcinomas have both receptors positive whilst 25% are lacking in them [8-12]. The ER+/PR- phenotype represents a distinct group because it usually is accompanied by high clinical and biological aggressiveness and have the least satisfactory response to hormone therapy in comparison to other phenotypes [13-16].

Regarding the phenotypes with one positive hormonal receptor (ER+/PR- and ER-/PR+) we noticed that they had the least number of cases, at total of 11 cases (11%) being recorded for the first phenotype and 5 for the later (5%).

Dividing the analysed cases according to their Her2 status was definitive following in situ hybridization and included 12 her2 positive cases (12%) and 88 Her2 negative cases (88%), placing them within the 10-34% interval of all mammary carcinomas that had an over-expression of the HER2 receptor and that are usually associated with a poor prognosis [17-21].

In the study by Freedman GM it was observed that patients with triple negative breast cancer have a high risk to develop local reoccurrences after 5 years [22-25], compared to other clinical and morphological factors (young age, resection margins and extensive intraductal component) as shown by the same author in a past study, represents a more reliable factor for predicting high rate of local reoccurrences [23]. Whilst some studies agree with Freedman, others have shown that there is a high risk of local reoccurrences for the *basal-like* subtype [26-29].

In regard to hormonal phenotypes we report that tumours with both hormonal receptors positive are more likely Her2 negative then Her positive (97.21% vs 2.79%). Tumours with absent hormonal receptors were Her2 negative in 86.33% of cases and Her2 positive ion 13.67% of cases. Mammary carcinoma that were solely lacking in PR receptors (ER+/PR- phenotype) were equally Her2 negative and Her2 positive (50% vs 50%), whilst those lacking in ER receptors (ER-/PR+) were 100% Her2 negative. Statistical analyses showed that there is a highly significant correlation between Her2 status and hormonal phenotypes (p=0.00063, VHS), Her2 negative tumours being typical for the classical phenotype whilst her2 positive tumours being more likely linked to the phenotype lacking in PR receptors.

Ipsilateral reoccurrences were statistically more significant more frequent at patients with absent oestrogen receptors compared with those who has these receptors present in the initial tumour (p=0.022). Therefore reoccurrences were more frequent if tumours were negative for estrogen-receptors compared to those who were ER+ (9 cases vs 6 cases, summing 26.47% vs 9.09%). Similarly it was observed that breast tumours with absent progesterone receptors reoccurred more frequently compared to those with present progesterone receptors (9 cases vs 6 cases, summing 21.42% of cases vs 10.34%) the difference being statistically significant (p=0.041).

In his study Martelli (2008) noticed that ipsilateral reoccurrences after conservative breast surgery are significantly associated with the status of oestrogen and progesterone receptors, finding more reoccurrence in women with ER positive tumours and PR positive tumours. Also, results from the same author show that for elderly patient, negative ER status is a predictive factor for metastasis and high mortality secondary to breast cancer, whilst for patient with favourable hormonal status the rate of survival until reoccurrence is much higher [30-33].

Similarly to our results, Komoiko showed in 2006 that factors significantly associated with rate of reoccurrence are absence of progesterone receptors, young age at first surgical intervention, size of the tumour, positive resection margins, high nuclear degree, presence of an extensive intra-ductal component together with the absence of endocrine therapy and post-surgical radiotherapy [34].

Another series of studies showed that ER negative tumours have a significantly higher risk of locally reoccurring after conservative breast surgery and radiotherapy [35-38] indicating that ER negativity in itself does not appear to be associated with a higher risk of local reoccurrence.

When talking about the hormonal immuno-phenotypes it was n noticed that the most frequent reoccurrences are associated to the phenotype with both receptors absent (ER-/PR-), followed by the phenotype with both receptors positive (ER+/PR+) while the phenotypes with one of the receptors absent showed no reoccurrences in our study. The correlation between the ipsilateral reoccurrence and hormonal phenotypes was statistically significant (0=0.033), the phenotype lacking in both receptors being the one that showed relapses most frequently.

Analysing the link between the Her2 status and the ipsilateral local reoccurrences we remark that these were almost 3 times more likely to occur for Her2 positive carcinomas compared to her2 negative ones (33.33% for all cases vs 11.36% of cases). Still comparing the reoccurrences for the cohort of Her2 positive patients with those shown in her 2 negative patients the results were not statistically significant (0.288), these being similar to

those presented by Freedman GM and Co in 2009, showing that her2 positive patients do not associate a higher rate of local reoccurrences compared to other patients. In the study done by Freedman GM and co in 2009, authors have shown that patients with ER -, PR- and Her2 positive tumours, treated conservatively, had the highest risk of developing metastasis and the lowest disease-free interval. Also authors have concluded, following follow-up of patients, that it was beneficial for their patients with triple negative tumours to undergo conservative surgery combined with radiotherapy.

The very recent study of Ishitobi M and Co in 2012 shows similar results to the present study , the authors commenting that none of their patients that underwent conservative breast surgery and had ER+ and Her2- did not develop any ipsilateral tumour reoccurrence for the period of the study and the frequency of the reoccurrences for this group of patients was significantly lower than those with different ER and Her2 status [39,40]. The only difference between our study and the one cited above was that the patients in the study done by Ishitobi M had pre-operatory neo-adjuvant chemotherapy. The authors have even proposed a new prognostic index of ipsilateral local tumour reoccurrences based on two factors identified as being predictive for these reoccurrence namely ER status and the multi-focal aspect of the residual tumour. Also, the same authors did not identify any statistically significant differences between the lot of her2 positive and her2 negative patients ($p=0.1889$) in regards to 4 year survival free of ipsilateral local tumour reoccurrence (IBTER).

Conclusions

The Her2/neu status detected though IHC correlates in a statistically significant manner with the absence of PR receptors and there is no statistical significant correlation evident between Her2/neu status detected though IHC and the absence of ER receptors.

Her2/neu status detected though CISH is highly and significantly correlating ($p=0.00063$, VHS) with hormonal phenotypes, Her2 negative tumours being typical for the classical immune-phenotype ER+PR+, whilst Her2 positive tumours are characteristic for the ER+PR- phenotype.

Her2 status after IHC and in situ hybridization is correlated significantly from a statistic point of view with the degree of differentiation of the carcinomas involved in our study ($p=0.033$), Her2 positive tumours being G3 poorly differentiated and not correlated to the stage of carcinoma at the time of diagnosis.

Local ipsilateral reoccurrences after conservative breast surgery are three times more likely to occur for carcinoma with her2 positive status compared to Her2 negative tumours, but again this was not statistically significant.

Ipsilateral reoccurrence following conservative breast surgery and radio-therapy is significantly associated with the absence of oestrogen receptors ($p=0.022$) and , the absence of progesterone receptors ($p=0.043$) as well as the phenotype negative for both receptors ER-PR- ($p=0.036$).

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