Histol Histopathol (2024) 39: 153-164

Histology and Histopathology
From Cell Biology to Tissue Engineering

ORIGINAL ARTICLE Open Access

Predictive markers for pathological complete response (pCR) after neo-adjuvant chemotherapy in HER2-positive breast carcinoma

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Summary. Objectives. Patients with HER2-positive invasive breast cancer that is node-positive and/or larger than 3 cm are generally treated with neoadjuvant chemotherapy (NAC). We aimed to identify predictive markers for pathological complete response (pCR) after NAC in HER2-positive breast carcinoma.

Methods. Hematoxylin/eosin-stained slides of 43 HER2-positive breast carcinoma biopsies were histopathologically reviewed. Immunohistochemistry (IHC) was performed on pre-NAC biopsies, comprising HER2, estrogen receptor (ER), progesterone receptor (PR), Ki-67, epidermal growth factor receptor (EGFR), mucin-4 (MUC4), p53 and p63. Dual-probe *HER2* in situ hybridization (ISH) was performed to study the mean *HER2* and *CEP17* copy numbers. ISH and IHC data were retrospectively collected for a validation cohort, comprising 33 patients.

Results. Younger age at diagnosis, 3+ HER2 IHC scores, high mean *HER2* copy numbers and high mean *HER2/CEP17* ratios were significantly associated with an increased chance of achieving a pCR, and the latter two associations were confirmed in the validation cohort. No other immunohistochemical or histopathological markers were associated with pCR.

Conclusions. This retrospective study of two community-based NAC-treated HER2-positive breast cancer patient cohorts identified high mean *HER2* copy numbers as a strong predictor for pCR. Further studies on larger cohorts are required to determine a precise cut-point for this predictive marker.

Key words: HER2 amplification, HER2 copy number, Pathological response, Neoadjuvant chemotherapy, Invasive breast carcinoma, Predictive markers

Introduction

HER2-positive breast cancer patients with clinically node-positive disease and/or tumors larger than 3 cm are generally treated with neoadjuvant chemotherapy (NAC) in combination with anti-HER2 targeted therapy (Korde et al., 2021). This treatment comprises either an anthracycline-based chemotherapy or non-anthracycline chemotherapy regimen, in combination with trastuzumab, with or without pertuzumab (Korde et al., 2021). NAC allows for an evaluation of the therapeutic response of the tumor, which is an important prognostic marker. For instance, an axillary pCR in HER2-positive breast cancer patients with cytologically proven axillary lymph node metastases is associated with excellent 10-year overall survival (Mougalian et al., 2016). Additionally, NAC can enable more conservative

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www.hh.um.es DOI: 10.14670/HH-18-626

Abbreviations. EGFR, epidermal growth factor receptor; ER, estrogen receptor; IHC, immunohistochemistry; ISH, in situ hybridization; MUC4, mucin 4; NAC, neoadjuvant chemotherapy; NST, no special type; pCR, pathological complete response; PR, progesterone receptor; RCB, residual cancer burden; SD, standard deviation

surgery by reduction of the tumor size (Derouane et al., 2022) although breast-conserving surgery after NAC is associated with a higher local recurrence risk (Asselain et al., 2018). The lack of therapeutic response to neoadjuvant systemic therapy is a strong predictor of poor breast cancer-specific survival, regardless of the molecular subtype, and therefore guides the subsequent adjuvant therapy (von Minckwitz et al., 2019; Korde et al., 2021; Yau et al., 2022).

De-escalation of subsequent adjuvant chemotherapy is currently being explored for HER2-positive breast cancer patients who achieved a pCR after decreased neoadjuvant treatment (Debien et al., 2022; Waks et al., 2022), as the risk of short- and long-term toxicity is one of the major disadvantages of both NAC and adjuvant chemotherapy (Derouane et al., 2022). Nevertheless, there is a lack of reliable markers to predict which breast cancer patients are most likely to achieve a pCR after standard-of-care NAC. Those patients may participate in ongoing and future clinical trials exploring the possibility of de-escalation of NAC regimens. Several potentially predictive markers for post-NAC pCR in HER2-positive breast cancer have been studied, especially in randomized clinical trial settings (Derouane et al., 2022). These predictive markers included both morphological features, such as tumor-infiltrating lymphocytes (TILs), and immunohistochemical markers, such as p53 protein expression, often with contradictory results. For instance, TILs were reported to be significantly associated with pCR in HER2-positive breast cancer patients (Denkert et al. 2018), but this was not observed in the NeoSphere and GeparSepto trials (Bianchini et al., 2015; Loibl et al., 2017). According to the Expert Panel of the American Society of Clinical Oncologists (ASCO), there is insufficient evidence at present to justify the routine use of potentially predictive markers to guide NAC regimens, regardless of the breast cancer molecular subtype (Korde et al., 2021).

We therefore aimed to retrospectively explore the potential predictive value of a series of histopathological and immunohistochemical markers for pCR in a consecutive, community-based HER2-positive breast cancer cohort, outside the clinical trial setting. We performed an in-depth histopathological review of pre-NAC biopsies, as well as dual-probe ISH analysis to study the mean *HER2* and *CEP17* copy numbers. The expression of estrogen receptor (ER), progesterone receptor (PR), Ki-67, and HER2 is routinely evaluated as it determines the surrogate molecular subtype, and these archived slides were reviewed. Additionally, we selected epidermal growth factor receptor (EGFR), mucin-4 (MUC4), p53 and p63 as immunohistochemical markers. EGFR was chosen because its overexpression in HER2-positive breast carcinomas is associated with poor disease-free survival and poor response to treatment with trastuzumab (Lee et al., 2015). MUC4 was selected because *in vitro* experiments showed that TNFα-induced MUC4 overexpression is a cause of trastuzumab resistance, and MUC4 overexpression was associated with poor disease-free survival in one retrospectively studied patient cohort (Mercogliano et al., 2017). Mutations in the *TP53* gene, encoding for the p53 protein, are frequently observed in triple-negative and HER2-positive breast carcinomas (Darb-Esfahani et al., 2016). *TP53* mutations were associated with pCR in a Chinese HER2-positive breast cancer cohort (Li et al., 2020), but this was not confirmed in the phase 2 GeparSixto trial (Darb-Esfahani et al., 2016). The myoepithelial marker p63 was chosen because its expression in HER2-positive breast cancer seems to be associated with abnormal p53 expression patterns, younger patient age and high histological grade (Guo et al., 2021). All morphological and immunohistochemical features were correlated with the subsequent therapeutic response in univariable and multivariable analyses, and the subsequent statistically significant associations were assessed in a second, independent HER2-positive breast cancer patient cohort.

Materials and methods

Patients and tissue samples in the study cohort

Tissue samples included in this study were core biopsies from a consecutive series of patients who were diagnosed with HER2-positive breast cancer between 1 January 2015 and 1 March 2020, and who were treated with NAC. The standard NAC regimen for HER2-positive breast cancer included either anthracycline/taxane or docetaxel/carboplatin in combination with trastuzumab, with or without pertuzumab (Korde et al., 2021). The subsequent surgery comprised breast-conserving surgery or mastectomy, either with sentinel lymph node procedure or axillary lymph node dissection depending on the initial clinical staging. Only those patients whose biopsy and resection specimen were both present in the archives of the Department of Pathology of the Cliniques universitaires Saint-Luc (Brussels, Belgium) were included in this study. Some patients received chemotherapy in outpatient clinics and surgery was performed in two different hospitals: therefore, data on chemotherapy regimen deviations and initial staging were not collected. Information on patient age at diagnosis, type of surgery, interval between the biopsy and the surgery, macroscopic tumor bed size, post-NAC TNM stage, and mean *HER2* and *CEP17* copy numbers was retrieved from electronic histopathological reports. This retrospective study was approved by the institutional ethics committee (file name: RETRO-HER2-15) and was performed in accordance with the World Medical Association's Declaration of Helsinki.

Tissue handling procedures and histopathological review

Breast biopsy cores were instantly fixed in 10% neutral-buffered formalin for 6-72h. All surgical specimens were received freshly, cut at 5 mm intervals, and fixed in 10% neutral-buffered formalin for 6-72h, following the ASCO/CAP guidelines (Hammond et al., 2010). The resection specimens were macroscopically

examined and extensively sampled according to the MD Anderson residual cancer burden (RCB) protocol (Symmans et al., 2007).

Archived hematoxylin and eosin (HE) stained slides of the pre-NAC core biopsies were retrieved and reviewed by one pathologist (MRVB). Tumors were graded and each component of the Nottingham grade was noted separately (Elston and Ellis, 1991). Mitoses were counted in ten high-power fields (HPF; i.e. at 400x magnification) using a light microscope with a field diameter of 0.55mm, corresponding to the following cut-points: low (≤8 mitoses), moderate (9-17 mitoses), and high (≥18 mitoses). The presence or absence of the following histopathological characteristics was dichotomously evaluated, regardless of its extent: any in situ component, morphological clear cell changes (i.e. tumor cells with clear cytoplasm), myxoid peritumor stroma (i.e. myxoid stromal changes), tumor necrosis, and unequivocal lympho-vascular invasion (Van Bockstal et al., 2020). Stromal tumor-infiltrating lymphocytes (TILs) were evaluated as percentages by three independent observers (MRVB, HD, CG), according to the International TILs Working Group 2014 methods (Salgado et al., 2015). Since TILs evaluation is prone to inter-observer variability (O'Loughlin et al., 2018; Kilmartin et al., 2021; Van Bockstal et al., 2021), the arithmetic mean of the three TILs scores was determined and used in subsequent statistical analyses. TILs were also dichotomized as low (≤40%) versus high (>40%), as previously described (Van Bockstal et al., 2021).

One pathologist (MRVB) reviewed the HE stained slides of all post-NAC resection specimens and determined the RCB score and RCB class of therapeutic response by using an online calculator (http://www3.mdanderson.org/app/medcalc/index.cfm?pagename=jsconvert3) (Symmans et al., 2007). An RCB score equaling zero (class RCB-0) was regarded as a pCR (Symmans et al., 2007).

Immunohistochemistry

Archived slides stained for ER, PR, HER2 and Ki-67 were reviewed by one pathologist (MRVB). Details on the antibodies and staining protocols are shown in Table 1. ER and PR expression were quantified by applying the Allred score (Harvey et al., 1999). A proportion score of <1% was regarded as negative, a proportion score of 1-10% was considered as weakly positive and a proportion score of >10% was designated as positive, in line with the ASCO/CAP guidelines for hormone receptor testing (Allison et al., 2020). The 2018 ASCO/CAP guidelines were used for assessment of HER2 protein expression as 0/negative, 1+/negative, 2+/equivocal, or 3+/positive (Wolff et al., 2018b). Ki-67 immunoreactivity was evaluated as the percentage of tumor nuclei showing positive staining, regardless of the intensity, in accordance with the recommendations of the International Ki67 in Breast Cancer Working Group (Nielsen et al., 2021).

Immunohistochemistry for EGFR, p53 and p63 was performed on the biopsy specimens, using an automated slide stainer (Benchmark XT, Ventana Medical Systems) according to the manufacturer's instructions (Table 1). Stained slides were evaluated jointly by two observers using a multi-head light microscope (MRVB, CG). Immunoreactivity in the entire biopsy was evaluated, regardless of the amount of tumor present. EGFR membrane staining was assessed according to the criteria for HER2 staining, and noted as 0/negative, 1+/negative, 2+/equivocal, or 3+/positive. In further statistical analyses, EGFR immunoreactivity was dichotomized as negative (score 0) versus any staining (scores 1+/2+/3+).

Membrane and cytoplasmic immunoreactivity for MUC4 was noted as a percentage of positive tumor cells. Tumors with ≥5% positive cells were regarded as MUC4-positive. P53 expression was evaluated as wild-type or mutation-type staining: the latter included total absence of immunoreactivity, overexpression (defined as intense nuclear staining in ≥70% of the neoplastic cells), or cytoplasmic staining, in line with the immunoreactivity patterns for high-grade serous tubo-ovarian carcinomas and endometrial carcinomas (Köbel et al., 2018). Nuclear p63 expression was evaluated as the percentage of positive tumor cells. Tumors with ≥5% positive cells were regarded as p63-positive. The

Table 1. Materials used for the immunohistochemical stains[a].

Protein	Clone	Firm	Species	Dilution	HIER	Visualisation
EGFR	31G7	Merck Millipore	Mouse	1/100	Protease K	UltraView DAB Detection[b]
ER	SP1	Roche	Rabbit	RTU	CC1	UltraView DAB Detection[b]
HER2	4B5	Roche	Rabbit	RTU	CC1	UltraView DAB Detection[b]
Ki67	MIB-1	Dako	Mouse	1/90	CC1	UltraView DAB Detection[b]
MUC4	8G7	Merck Millipore	Mouse	1/60	CC2	UltraView DAB Detection[b]
p53	DO-7	Biocare Medical	Mouse	1/200	CC1	UltraView DAB Detection[b]
p63	4A4	Bio SB	Mouse	1/50	CC1	UltraView DAB Detection[b]
PR	1E2	Roche	Rabbit	RTU	CC1	UltraView DAB Detection[b]

CC1, Cell Conditioning 1 Tris-based buffer[b]; CC2, Cell conditioning 2 buffer[b]; EGFR, epidermal growth factor receptor; ER, estrogen receptor; HIER: heat-induced epitope retrieval; IHC, immunohistochemistry; PR, progesterone receptor; RTU, ready-to-use. [a]: Performed on 4-μm-thick sections mounted on Superfrost plus slides (Menzel-Gläser, Braunschweig, Germany). [b]: Ventana Medical Systems, Tucson, Arizona (USA).

presence of an in situ component was confirmed by the identification of p63-positive myoepithelial cells.

Dual-probe HER2 in situ hybridization (ISH)

The amplification status of all tumors was investigated by ISH using the INFORM HER2 Dual ISH Cocktail probe on an automated slide stainer, according to the manufacturer's instructions (Benchmark XT, Ventana Medical Systems, Arizona, USA). Both the mean *HER2* and mean *CEP17* copy numbers were noted, and the *HER2/CEP17* ratio was calculated. Prior to 1 November 2018, the amplification status was assessed following the 2013 ASCO/CAP guidelines, and patients were treated accordingly (Wolff et al., 2013). Core biopsies obtained after 1 November 2018 were assessed according to the 2018 ASCO/CAP guidelines (Wolff et al., 2018a), and patients were treated accordingly. This change in the ASCO/CAP guidelines only influenced the eligibility of patients for neoadjuvant anti-HER2 targeted treatment at the time of their diagnosis. The changed guidelines did not modify the way the ISH technique was performed, and therefore, the determination of the mean HER2 and CEP17 copy numbers and the HER2/CEP17 ratio were not influenced.

Patients in the validation cohort

The validation cohort comprised NAC-treated HER2-positive breast cancer patients who underwent surgery between 20 December 2020 and 30 September 2022. Surgery included breast-conserving surgery or mastectomy, either with sentinel lymph node procedure or axillary lymph node dissection depending on the initial clinical staging. Information on patient age at diagnosis, interval between the biopsy and the surgery, post-NAC TNM stage, histological subtype, RCB class, RCB score, Nottingham grade, ER and PR status, Ki-67 expression, HER2 IHC and mean *HER2* and *CEP17* copy numbers was retrieved from electronic histopathological reports at the Cliniques universitaires Saint-Luc (Brussels, Belgium). This retrospective study was approved by the institutional ethics committee (file name: RETRO-ER-PR-RATES) and was performed in accordance with the World Medical Association's Declaration of Helsinki.

Statistical analysis

Statistical analyses were conducted using IBM SPSS statistics 27.0 software (Chicage, IL, USA). pCR (i.e. class RCB-0) was the outcome variable. Continuous variables were tested for normal distribution using the Shapiro-Wilk test. Normally-distributed data were tested with the Levene's test for equality of variances. Normally-distributed data with equal variances in the study cohort comprised patient age at diagnosis (in years) and the interval between the biopsy diagnosis and surgery (in months). Normally-distributed data with equal variances in the validation cohort comprised Ki-67 expression, mean *HER2* copy numbers, mean *HER2/CEP17* ratios and patient age at diagnosis (in years). These variables were analyzed with the Student t-test and reported as mean ± standard deviation (SD). All other continuous variables in the study cohort and the validation cohort were not normally-distributed and were analyzed with the Mann-Whitney U test.

Categorical data were analyzed with the Chi-Square test or Fisher's Exact test when appropriate, and were reported as absolute number with relative percentage. Multivariable logistic regression analysis with backward selection was conducted in the study cohort to determine which features were independently associated with pCR, while taking into account related variables because of multicollinearity. All tests were two-sided, with the statistical significance level set at p=0.05.

Results

Clinicopathological features and pCR in the study cohort

Table 2 provides an overview of the patient characteristics in the study cohort. The average patient age at diagnosis was 57 (range 31.5-80.2 years). The average time between the biopsy diagnosis and subsequent post-NAC surgery was 6.2 months (range 4.0-7.8 months). All patients were diagnosed with a HER2-positive tumor, confirmed by dual-probe ISH. Each tumor included in the study cohort qualified as a 'group 1' HER2-positive carcinoma (i.e. *HER2/CEP17* ratio ≥2.0 and mean *HER2* copy number ≥4.0) according to the ASCO/CAP guidelines (Wolff et al., 2018b). Six patients had a special type invasive carcinoma, comprising one metaplastic (squamous) carcinoma, one mucinous carcinoma, two carcinomas of no special type (NST) admixed with micropapillary carcinoma, and two pleomorphic lobular carcinomas. Seventeen patients (40%) achieved a pCR.

None of the clinical and histopathological characteristics was associated with pCR, except for age at diagnosis (Tables 3, 4): patients who achieved a pCR were significantly younger (p=0.033).

Immunohistochemistry and pCR in the study cohort

Immunohistochemistry for p63 was available for 41 out of 43 cases (95%), and immunohistochemistry for EGFR, p53 and MUC4 was available for 40 out of 43 cases (93%). Missing data were due to tissue block exhaustion. No immunohistochemical characteristics were significantly associated with pCR, neither as continuous (Table 3) nor as categorical variable (Table 5), with the exception of HER2 immunohistochemistry (Fig. 1). All but one patient with a pCR (94%) had a tumor with a 3+ HER2 IHC score, whereas only 12 out of 26 patients without pCR (46%) had a 3+ score (p=0.005). When PR expression was dichotomized as

negative (<1%) versus weakly positive or positive (≥1%), a slightly higher proportion of pCR was observed among the PR-negative tumors but this was not statistically significant (p=0.053). EGFR expression, p53 mutation-type staining and p63-positivity in HER2-positive breast carcinomas were not associated with an increased chance of achieving a pCR (p>0.05).

Table 2. Patient characteristics and biopsy-based histopathological features of the study cohort containing 43 HER2-positive carcinoma patients, and in the validation cohort containing 33 HER2-positive carcinoma patients.

	Study cohort Mean ± SD	Validation cohort Mean ± SD
Age at biopsy-based diagnosis (years)	57.2±13.0	51.5±12.8
Interval between biopsy and surgery (months)	6.2±1.0	5.2±1.0
	n (%)	n (%)
Sex		
Male	0 (0)	0 (0)
Female	43 (100)	33 (100)
Type of surgery		
Lumpectomy	16 (37)	14 (42)
Quadrantectomy	3 (7)	5 (15)
Mastectomy	24 (56)	14 (42)
Laterality		
Left breast	20 (47)	15 (45)
Right breast	23 (53)	18 (55)
Cancer type		
Invasive carcinoma of NST	37 (86)	31 (94)
Special type invasive carcinoma	6 (14)	2 (6)
Nottingham grade		
Grade 1	0 (0)	0 (0)
Grade 2	19 (44)	13 (39)
Grade 3	24 (56)	20 (61)
Lympho-vascular invasion		
Absent	37 (86)	29 (88)
Present	6 (14)	4 (12)
Ductal carcinoma in situ component		
Absent	35 (81)	25 (76)
Present	8 (19)	8 (24)
RCB class		
Class RCB-0 (pCR)	17 (40)	21 (64)
Class RCB-I	10 (23)	4 (12)
Class RCB-II	11 (26)	7 (21)
Class RCB-III	5 (12)	1 (3)
ER immunohistochemistry		
Negative (< 1%)	14 (33)	12 (36)
Weakly positive (1-10%)	4 (9)	2 (6)
Positive (> 10%)	25 (58)	19 (58)
PR immunohistochemistry		
Negative (< 1%)	20 (47)	19 (58)
Weakly positive (1-10%)	6 (14)	2 (6)
Positive (> 10%)	17 (40)	12 (36)
HER2 positivity status		
1+ or 2+ with HER2 amplification	15 (35)	5 (15)
3+ with HER2 amplification	28 (65)	28 (85)

ER, estrogen receptor; NA, not assessed; NST, no special type; pCR, pathological complete response; PR, progesterone receptor; RCB, residual cancer burden; SD, standard deviation

ISH and pCR in the study cohort

Both the mean *HER2* copy number and the *HER2/CEP17* ratio were significantly associated with pCR (Table 3, Fig. 2), which is in line with the significant association between HER2 IHC and pCR. Tumors with a post-NAC pCR showed substantially higher mean *HER2* copy numbers and higher mean *HER2/CEP17* ratios (p=0.005 and p=0.001, respectively). The mean *CEP17* copy number was not significantly associated with pCR (p=0.080), although there was a subtle tendency towards higher *CEP17* copy numbers in tumors without pCR.

Multivariable logistic regression analysis with backward selection was conducted to determine which features were independently associated with pCR. Mean *HER2* copy number, mean *HER2/CEP17* ratio and HER2 IHC were introduced separately in these models, as these variables are highly related. None of the other clinical, histopathological or immunohistochemical markers was significantly associated with pCR in the various multivariable models. After correction for age at diagnosis, both mean *HER2* copy number and mean *HER2/CEP17* ratio remained significantly associated with pCR (p=0.023 and p=0.011, respectively).

Predictive factors for pCR in the validation cohort

We aimed to validate our observations regarding the predictive value of patient age, HER2 immunohistochemistry, mean *HER2* copy number and mean *HER2/CEP17* ratio in an independent patient cohort,

Table 3. Study cohort. Clinicopathological and immunohistochemical differences (continuous variables) between HER2-positive breast carcinomas with (n=17) and without (n=26) pathological complete response (pCR).

	pCR Mean ± SD	No pCR Mean ± SD	p-value
Age at diagnosis (years)	51.0±11.6	59.8±13.1	0.033[a]*
Interval biopsy/surgery (months)	6.22±0.87	6.20±1.10	0.957[a]
Mitotic count	13±13	18±15	0.218[b]
TILs (%) – observer A	36±31	26±28	0.348[b]
TILs (%) – observer B	36±29	22±25	0.072[b]
TILs (%) – observer C	38±26	31±25	0.209[b]
TILs (%) – mean score three observers	37±28	27±25	0.129[b]
ER (%)	38±39	55±40	0.244[b]
PR (%)	15±32	22±29	0.071[b]
Ki-67 (%)	54±22	58±27	0.329[b]
p63 (%)	3±4	6±15	0.816[b]
Mean *HER2* copy number	15.66±4.50	11.91±5.79	0.005[b]*
Mean *CEP17* copy number	1.95±0.34	2.29±0.63	0.080[b]
Mean *HER2/CEP17* ratio	8.23±2.80	5.57±3.00	0.001[b]*

CEP17, chromosome enumeration probe 17; ER, estrogen receptor; pCR, pathological complete response; PR, progesterone receptor; SD, standard deviation; TILs, tumor-infiltrating lymphocytes. [a]: Student t test for normally distributed data, with equal variances confirmed by Levene's test. [b]: Mann-Whitney U-test for non-normally distributed data. *: Statistically significant result (α=0.05).

containing 33 patients with NAC-treated HER2-positve breast cancer. Table 2 shows the patient characteristics in the validation cohort. Two patients had a special type invasive carcinoma, comprising one invasive mucinous carcinoma and one mixed NST/invasive lobular carcinoma. All other patients had NST invasive breast cancer. The average age at diagnosis was 51.5 (range 31-80 years). The average time between the biopsy diagnosis and subsequent post-NAC surgery was 5.2 months (range 2-7 months). All but two tumors included in this validation cohort qualified as a 'group 1' HER2-positive carcinoma (i.e. *HER2/CEP17* ratio ≥2.0 and mean *HER2* copy number ≥4.0) according to the ASCO/CAP guidelines (2018 guidelines). Two tumors qualified as a 'group 3' HER2-positive carcinoma (i.e. *HER2/CEP17* ratio <2.0 and mean *HER2* copy number ≥6.0). ISH data were missing for 5 patients with a 3+ HER IHC score, who had had their biopsy elsewhere.

Both higher mean *HER2* copy number and higher mean *HER2/CEP17* ratio were significantly associated with a higher chance of achieving a pCR (p=0.015 and p=0.033, respectively; Table 6). Only five out of 33 patients had a 2+ HER2 IHC score; two achieved a pCR

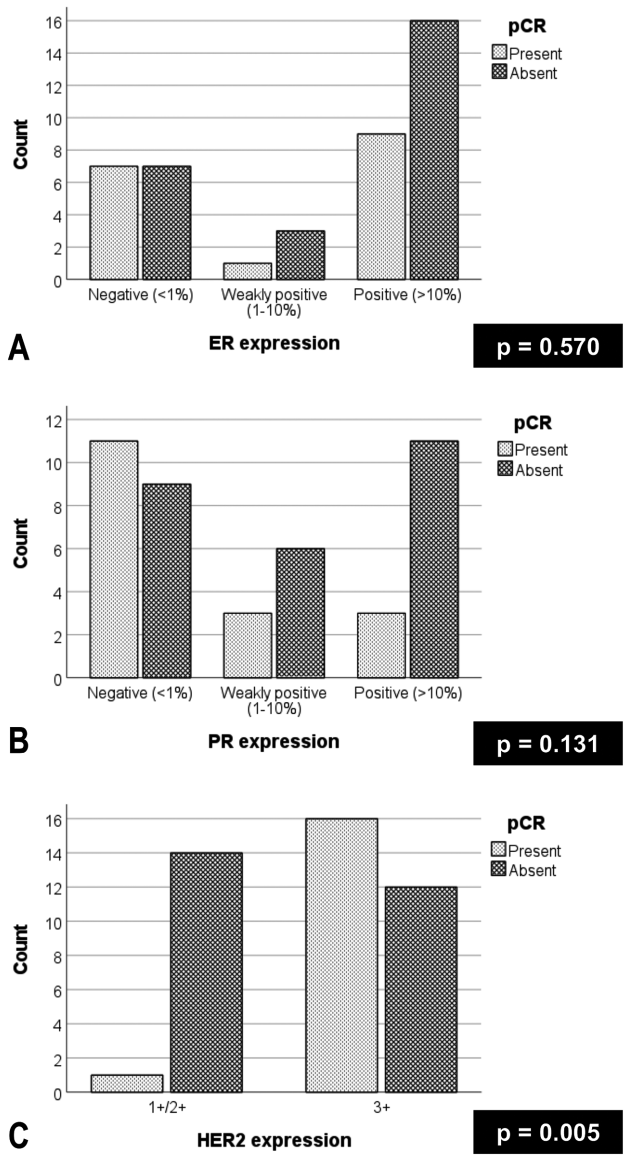

Fig. 1. Bar charts illustrating the relationship between pCR and ER expression **(A)**, PR expression **(B)** and HER2 immunohistochemistry **(C)**.

Table 4. Study cohort. Clinicopathological differences (categorical variables) between HER2-positive breast carcinomas with (n=17) and without (n=26) pathological complete response (pCR).

	pCR n (%)	No pCR n (%)	p-value[a]
Type of surgery			0.759
Breast-conserving surgery	8 (47)	11 (42)	
Mastectomy	9 (53)	15 (58)	
Cancer type			0.217
NST	16 (94)	21 (81)	
Special type	1 (6)	5 (19)	
Nottingham grade			0.759
Grade 1	0 (0)	0 (0)	
Grade 2	8 (47)	11 (42)	
Grade 3	9 (53)	15 (58)	
Glandular differentiation			0.342
>75%	0 (0)	0 (0)	
≤75% and ≥10%	1 (6)	4 (15)	
<10%	16 (94)	22 (85)	
Nuclear atypia			0.532
Low	0 (0)	0 (0)	
Moderate	1 (6)	3 (13)	
High	16 (94)	23 (87)	
Mitotic score			0.251
Low	8 (47)	6 (23)	
Moderate	5 (29)	10 (38)	
High	4 (24)	10 (38)	
Lympho-vascular invasion			0.738
Absent	15 (88)	22 (85	
Present	2 (12)	4 (15)	
DCIS			0.141
Absent	12 (71)	23 (89)	
Present	5 (29)	3 (12)	
Tumor necrosis			0.181
Absent	12 (71)	13 (50)	
Present	5 (29)	13 (50)	
Clear cell changes			0.084
Absent	9 (53)	7 (27)	
Present	8 (47)	19 (73)	
Myxoid stroma			0.480
Absent	6 (35)	12 (46)	
Present	11 (65)	14 (54)	
TILs (dichotomous evaluation)			0.383
0-40%	11 (65)	20 (77)	
>40%	6 (35)	6 (23)	

DCIS, ductal carcinoma in situ; NST, no special type; pCR, pathological complete response; TILs, tumor-infiltrating lymphocytes. [a]: Determined by Chi-Square test.

Table 5. Study cohort. Immunohistochemical differences (categorical variables) between HER2-positive breast carcinomas with (n=17) and without (n=26) pathological complete response (pCR).

	pCR n (%)	No pCR n (%)	p-value[a]
ER immunohistochemistry			0.570
Negative (<1%)	7 (41)	7 (27)	
Weakly positive (1-10%)	1 (6)	3 (12)	
Positive (>10%)	9 (53)	16 (62)	
PR immunohistochemistry			0.131
Negative (<1%)	11 (55)	9 (35)	
Weakly positive (1-10%)	3 (18)	6 (23)	
Positive (>10%)	3 (18)	11 (42)	
HER2 immunohistochemistry			0.005*
Score 0	0 (0)	0 (0)	
Score 1+	0 (0)	1 (4)	
Score 2+	1 (6)	13 (50)	
Score 3+	16 (94)	12 (46)	
Ki-67 immunohistochemistry			0.757
<20%	1 (6)	1 (4)	
≥20%	16 (94)	25 (96)	
p53 immunohistochemistry			0.505
Wild-type staining	11 (69)	14 (58)	
Mutated-type staining	5 (31)	10 (42)	
EGFR immunohistochemistry			0.107
Negative (score 0)	13 (81)	13 (57)	
Any staining (score 1+/2+/3+)	3 (19)	10 (43)	
MUC4 immunohistochemistry			0.262
Negative (<5%)	11 (73)	21 (88)	
Positive (≥5%)	4 (27)	3 (13)	
P63 immunohistochemistry			0.872
Negative (<5%)	13 (81)	19 (79)	
Positive (≥5%)	3 (19)	5 (21)	

EGFR, epidermal growth factor receptor; ER, estrogen receptor; pCR, pathological complete response; PR, progesterone receptor. [a]: Determined by Chi-Square test. *: Statistically significant result (α=0.05).

Table 6. Validation cohort. Clinicopathological and immunohistochemical differences (continuous and categorical variables) between HER2-positive breast carcinomas with (n=21) and without (n=12) pathological complete response (pCR).

Continuous variables	pCR Mean±SD	No pCR Mean±SD	p-value
Age at diagnosis (years)	48±10	59±15	0.116[a]
Interval biopsy/surgery (months)	5.38±0.62	4.70±1.34	0.307[b]
ER (%)	36±44	73±34	0.014[b]*
PR (%)	27±40	34±42	0.343[b]
Ki-67 (%)	42±20	36±18	0.190[a]
Mean *HER2* copy number	16.19±5.01	11.87±3.75	0.015[a]*
Mean *CEP17* copy number	2.48±0.64	2.68±0.67	0.324[b]
Mean *HER2/CEP17* ratio	6.71±2.18	4.79±2.18	0.033[a]*
Categorical variables	**pCR n (%)**	**No pCR n (%)**	**p-value**
Cancer type			0.125[c]
NST	21 (100)	10 (83)	
Special type	0 (0)	2 (17)	
Nottingham grade			0.840[c]
Grade 1	0 (0)	0 (0)	
Grade 2	8 (38)	5 (42)	
Grade 3	13 (62)	7 (58)	
HER2 immunohistochemistry			0.328[c]
Score 0	0 (0)	0 (0)	
Score 1+	0 (0)	0 (0)	
Score 2+	2 (10)	3 (25)	
Score 3+	19 (91)	9 (75)	

CEP17, chromosome enumeration probe 17; ER, estrogen receptor; pCR, pathological complete response; PR, progesterone receptor; SD, standard deviation. [a]: Student t test for normally distributed data, with equal variances confirmed by Levene's test. [b]: Mann-Whitney U-test for non-normally distributed data. [c]: Determined by Chi-Square test or Fisher's Exact test, when appropriate. *: Statistically significant result (α=0.05).

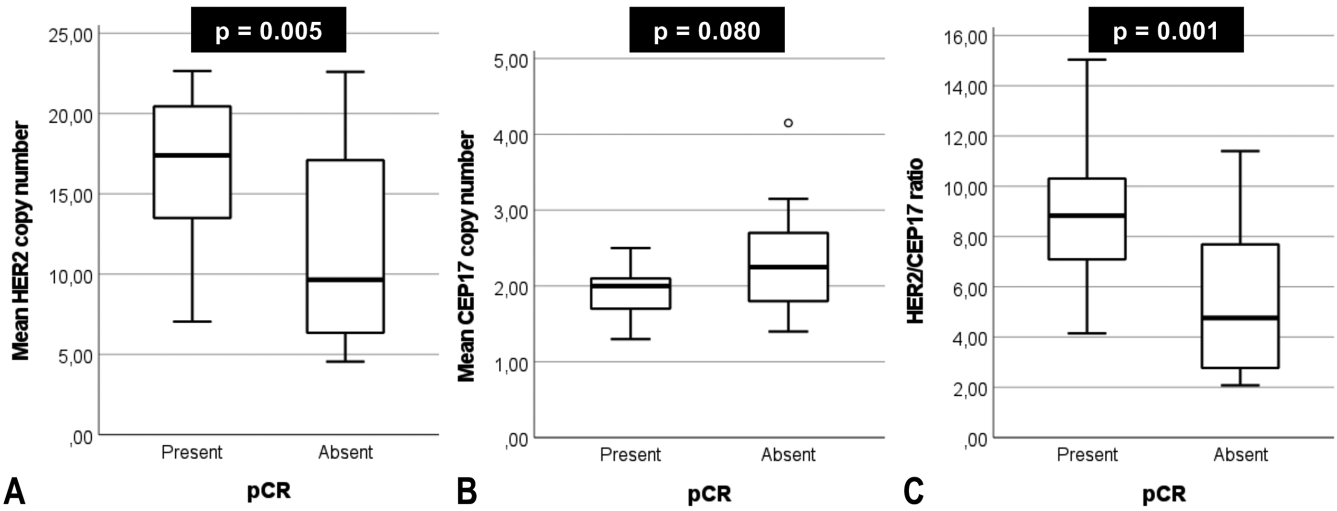

Fig. 2. Box-and-whisker plots illustrating the relationship between pCR and mean *HER2* copy number **(A)**, mean *CEP17* copy number **(B)** and mean *HER2/CEP17* ratio **(C)**.

and three did not. We were therefore not able to confirm the strong association between HER2 IHC score and pCR in the validation cohort. Although the mean age was younger in the pCR group than in the 'no pCR' group, this difference was not statistically significant. The pCR group of the validation cohort showed a substantially lower ER positivity rate than the 'no pCR' group (p=0.014). PR status, Ki-67 expression, mean *CEP17* copy number, histological subtype and Nottingham grade were not associated with pCR in the validation cohort.

Discussion

In this study, we demonstrated that high mean *HER2* copy numbers and high *HER2/CEP17* ratios are associated with a substantially higher chance to achieve a pCR after NAC in HER2-positive breast cancers. Our observations corroborate the conclusions of previous reports on the *HER2/CEP17* ratio as a predictor for pCR in HER2-positive breast cancer (Kogawa et al., 2016; Singer et al., 2017; Choi et al., 2020; Zhao et al., 2020; Antolín et al., 2021). However, three of these studies analyzed the *HER2/CEP17* ratio as a categorical variable, with thresholds varying from >5 to ≥7 to distinguish low level from high level amplification (Kogawa et al., 2016; Singer et al., 2017; Antolín et al., 2021). We preferred to study the *HER2/CEP17* ratio as a continuous variable, in order not to lose any information due to dichotomization. At present, there is insufficient evidence to support a particular threshold. The higher the *HER2/CEP17* ratio, the more likely a patient will achieve a pCR. Proposing a specific cut-off will first require a discussion on the number of false-positives (patients with a high *HER2/CEP17* ratio but without pCR) and false-negatives (patients with a low *HER2/CEP17* ratio but who do achieve a pCR) that oncologists find acceptable in routine practice. This is especially important if this molecular feature is to be used for selection of patients eligible for de-escalation of the current standard-of-care NAC regimens.

Similarly to the *HER2/CEP17* ratio, high mean HER2 copy numbers are associated with a larger chance of achieving a pCR, which was also shown by Antolin et al. (2021) and Zhao et al. (2020). Of note, Zhao et al. grouped RCB-0 and RCB-1 patients together as responders (Zhao et al., 2020), but we preferred to distinguish patients with a pCR from patients without a pCR, analogous to most studies investigating predictors for pCR (Symmans et al., 2007). Antolin et al. proposed a cut-off of ≤10 versus >10 mean *HER2* copies per nucleus to discern patients with low from high level amplification, which was associated with a 28% versus 61% pCR rate, respectively (Antolín et al., 2021). We believe it is more prudent at present to mention the mean *HER2* copy number as a continuous variable. The mean *CEP17* copy number was not associated with pCR in our cohort, as previously reported by Katayama et al. (Katayama et al., 2022).

Analogous to the ISH data, patients with a 3+ HER2 IHC score in the initial study cohort were much more likely to achieve a pCR than patients with a 2+ HER2 IHC score and *HER2* gene amplification, which corroborates the findings of others (Krystel-Whittemore et al., 2019; Zhao et al., 2020; Katayama et al., 2021). However, HER2 immunohistochemistry seems somewhat less refined than the mean *HER2* copy number to identify patients at high risk of being a non-responder to NAC. In our study cohort, 46% of patients without pCR still showed a 3+ IHC score, and this number rose to 75% in the non pCR group of our validation cohort. The mean *HER2* copy number seems more promising for identification of those patients who might be eligible for de-escalation regimens, although future studies should decide upon which threshold to use for this purpose.

But why is the mean *HER2* copy number so predictive for response to NAC, and thus, for pCR? We speculate that there are two potential causes. Firstly, IHC evaluates protein expression. Variations in fixation methods, fixation time and their impact on the antigenicity of the protein may render IHC evaluation less consistent (Sauter et al., 2009). ISH is somewhat less prone to fixation-related artefacts and might be more robust. ISH data correlate better with responsiveness to anti-HER2 targeted therapies, and Sauter et al. therefore advocated ISH as the primary HER2 testing modality (Sauter et al., 2009). Secondly, breast tumors harboring high *HER2* copy numbers might have more HER2 protein, rendering them more dependent or 'addicted' to this oncogene, and thus more sensitive to anti-HER2 targeted therapies. IHC might be insufficiently sensitive to distinguish breast tumors with large amounts of HER2 protein ('high expressors') from those tumors with much larger amounts of HER2 protein ('very high expressors'). The rationale for this explanation can be found in the 'gene dosage effect': in both amplified and non-amplified cancers, increased mean *HER2* copy numbers are associated with higher IHC scores (Lambein et al., 2011). In other words: the more HER2 copies, the more protein, the stronger the oncogene addiction and the more sensitive to NAC.

Overall, our study identified a limited number of promising predictive markers, which might be due to a lack of power, as the study cohort contained only 43 patients. However, statistically significant associations in extensive cohorts do not automatically reflect biologically relevant relations, as even very discrete differences can result in a low p-value, and therefore, a statistically significant association, if the study cohort is sufficiently large (Lovell, 2013). A potent predictor of pCR does not require a large study cohort, which was previously illustrated by the strong predictive power of TILs for achieving a pCR in a cohort of 35 patients with triple-negative breast cancer (Van Bockstal et al., 2020). Likewise, we were able to validate the findings of others regarding the predictive value of HER2 ISH data for pCR in two independent, real-life patient cohorts outside

the clinical trial setting, containing only 43 and 33 patients. This emphasizes the strong predictive power of both mean *HER2* copy number and mean *HER2/CEP17* ratio.

Younger patient age was associated with pCR in the study cohort, which was likely due to chance, as we were not able to validate this in the validation cohort. Studies on larger cohorts did not observe a substantial relation between younger age and higher chance of achieving a pCR (Singer et al., 2017; Krystel-Whittemore et al., 2019; Zhao et al., 2020; Katayama et al., 2021). We did not study other clinical parameters such as body mass index, nodal status or menopausal status, but others previously showed no association with pCR (Singer et al., 2017; Krystel-Whittemore et al., 2019).

As for the other histopathological features studied here, we did not observe any significant association with pCR. Again, this might have been caused by a lack of power, but a really strong discriminator would likely have revealed itself in this exploratory analysis, as was shown for *HER2* ISH data. We therefore deem it unlikely that histopathological features such as tumor grade or TILs are sufficiently robust for the identification of HER2-positive breast cancer patients with high likelihood of achieving a pCR, in contrast with triple-negative breast cancer patients. High tumor grade was only shown to be predictive for pCR in two large cohorts of 500 and 531 patients (Krystel-Whittemore et al., 2019; Katayama et al., 2021). Although TILs were predictive for pCR in a small series of triple negative breast cancer patients, despite the presence of substantial inter-observer variability (Van Bockstal et al., 2021), we were not able to confirm their predictive power in our study cohort of HER2-positive breast cancer patients. This is supported by the presently available data from several clinical trials. Although two pooled analyses showed that high baseline TILs were predictive for pCR in HER2-positive breast cancer (Solinas et al., 2017; Denkert et al., 2018), a TRYPHAENA sub-study, the NeoSphere trial and the GeparSepto trial were not able to confirm these observations (Bianchini et al., 2015; Loibl et al., 2017; Ignatiadis et al., 2019). In this study, we did not assess pre-NAC tumor stage or nodal status, but three retrospective studies were not able to show a significant association with the chance of achieving a pCR in HER2-positive breast cancer patients (Guiu et al., 2010; Singer et al., 2017; Krystel-Whittemore et al., 2019).

Regarding additional immunohistochemical markers to finetune the risk stratification of HER2-positive breast cancer patients receiving NAC, the evaluation of hormone receptor status seems most promising. Nevertheless, data on the predictive value of ER and PR are contradictory at present. In our own study cohort, we did not observe a relation between ER positivity and pCR, although there was a tendency towards slightly higher PR percentages in the non pCR group. However, in our validation cohort, we observed the opposite situation: there was no relation between PR expression and pCR, but ER expression levels were substantially lower in the pCR group than in the non pCR group. A similar relation between ER expression and pCR was observed by Katayama et al. in the ASCO/CAP ISH group 1 patients (Katayama et al., 2021). Krystel-Whittemore et al. observed a significant relation between both ER and PR expression and pCR in univariable analyses, but only the association between PR expression and pCR was maintained in multivariable analysis (Krystel-Whittemore et al., 2019). Both groups investigated large (≥500) patient cohorts, suggesting that there might be only a subtle relationship between ER/PR-negativity and higher chance of achieving a pCR at the population level. Hormone receptor status seems therefore less likely to be applicable at the individual patient level, as there is a huge overlap in expression rates among the pCR and non pCR groups.

We explored the potentially predictive value of some less well-known immunohistochemical markers. Immunoreactivity for EGFR, MUC4, p53 and p63 is unlikely to yield any predictive information, as we did not observe any association with pCR. We observed a p53 mutation-type staining in 35% of tumors, which is a lower frequency than that observed in triple-negative breast cancers (Darb-Esfahani et al., 2016; Van Bockstal et al., 2020).

In conclusion, we identified high mean *HER2* copy numbers and high mean *HER2/CEP17* ratios as strong predictors for pCR. These ISH data seem very promising for application in daily routine practice, as we were able to validate their predictive value in a second cohort. Both mean *HER2* copy number and mean *HER2/CEP17* ratio seem sufficiently robust for risk stratification at the individual patient level. Future studies should address whether these molecular features could be used to identify those patients who may be eligible for inclusion in NAC de-escalation trials.

Acknowledgements and funding sources. The authors would like to thank Mr. Philippe Camby and Ms. Martine Stevens for their excellent technical support for the immunohistochemistry used in this study.

This work was supported by a postdoctoral clinical mandate (2019-089 to M.R.V.B) from the not-for-profit organisation 'Foundation Against Cancer' (Brussels, Belgium).

Conflict of Interest. M.R.V.B. previously received research funding from Roche, as well as a reimbursement for being a member of an advisory board of AstraZeneca, all unrelated to the present work.

The other authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

References

Allison K.H., Hammond M.E.H., Dowsett M., McKernin S.E., Carey L.A., Fitzgibbons P.L., Hayes D.F., Lakhani S.R., Chavez-MacGregor M., Perlmutter J., Perou C.M., Regan M.M., Rimm D.L., Symmans W.F., Torlakovic E.E., Varella L., Viale G., Weisberg T.F., McShane L.M. and Wolff A.C. (2020). Estrogen and progesterone receptor testing in breast cancer: American Society of Clinical Oncology/College of

American Pathologists guideline update. Arch. Pathol. Lab. Med. 144, 545-563.

Antolín S., García-Caballero L., Reboredo C., Molina A., Mosquera J., Vázquez-Boquete Á., Gallego R., Santiago M.P., Concha Á., Pérez E., Calvo L. and García-Caballero T. (2021). Is there a correlation between HER2 gene amplification level and response to neoadjuvant treatment with trastuzumab and chemotherapy in HER2-positive breast cancer? Virchows Arch. 479, 853-857.

Asselain B., Barlow W., Bartlett J., Bergh J., Bergsten-Nordström E., Bliss J., Boccardo F., Boddington C., Bogaerts J., Bonadonna G., Bradley R., Brain E., Braybrooke J., Broet P., Bryant J., Burrett J., Cameron D., Clarke M., Coates A., Coleman R., Coombes R.C., Correa C., Costantino J., Cuzick J., Danforth D., Davidson N., Davies C., Davies L., Di Leo A., Dodwell D., Dowsett M., Duane F., Evans V., Ewertz M., Fisher B., Forbes J., Ford L., Gazet J.C., Gelber R., Gettins L., Gianni L., Gnant M., Godwin J., Goldhirsch A., Goodwin P., Gray R., Hayes D., Hill C., Ingle J., Jagsi R., Jakesz R., James S., Janni W., Liu H., Liu Z., Lohrisch C., Loibl S., MacKinnon L., Makris A., Mamounas E., Mannu G., Martín M., Mathoulin S, Mauriac L., McGale P., McHugh T., Morris P., Mukai H., Norton L., Ohashi Y., Olivotto I., Paik S., Pan H., Peto R., Piccart M., Pierce L., Poortmans P., Powles T., Pritchard K., Ragaz J., Raina V., Ravdin P., Read S., Regan M., Robertson J., Rutgers E., Scholl S., Slamon D., Sölkner L., Sparano J., Steinberg S., Sutcliffe R., Swain S., Taylor C., Tutt A., Valagussa P., van de Velde C., van der Hage J., Viale G., von Minkwitz G., Wang Y., Wang Z., Wang X., Whelan T., Wilcken N., Winer E., Wolmark N., Wood W., Zambetti M., Zujewski, J.A. (2018). Long-term outcomes for neoadjuvant versus adjuvant chemotherapy in early breast cancer: meta-analysis of individual patient data from ten randomised trials. Lancet Oncol. 19, 27-39.

Bianchini G., Pusztai L., Pienkowski T., Im Y.H., Bianchi G.V., Tseng L.M., Liu M.C., Lluch A., Galeota E., Magazzù D., de la Haba-Rodríguez J., Oh D. Y., Poirier B., Pedrini J.L., Semiglazov V., Valagussa P. and Gianni L. (2015). Immune modulation of pathologic complete response after neoadjuvant HER2-directed therapies in the NeoSphere trial. Ann. Oncol. 26, 2429-2436.

Choi J.H., Jeon C.W., Kim Y. O. and Jung S. (2020). Pathological complete response to neoadjuvant trastuzumab and pertuzumab therapy is related to human epidermal growth factor receptor 2 (HER2) amplification level in HER2-amplified breast cancer. Medicine 99, e23053.

Darb-Esfahani S., Denkert C., Stenzinger A., Salat C., Sinn B., Schem C., Endris V., Klare P., Schmitt W., Blohmer J.-U., Weichert W., Möbs M., Tesch H., Kümmel S., Sinn P., Jackisch C., Dietel M., Reimer T., Loi S., Untch M., von Minckwitz G., Nekljudova V. and Loibl S. (2016). Role of TP53 mutations in triple negative and HER2-positive breast cancer treated with neoadjuvant anthracycline/taxane-based chemotherapy. Oncotarget 7, 67686-67698.

Debien V., Adam V., Caparica R., Fumagalli D., Velghe C., Gaye J., de Nobrega V.C., Arahmani A., Zoppoli G. and Piccart-Gebhart M.J. (2022). DECRESCENDO: De-escalation of adjuvant chemotherapy in patients with HER2+/HR-/node-negative early breast cancer who achieve pCR after neoadjuvant taxane and subcutaneous dual anti-HER2 blockade. J. Clin. Oncol. 40, TPS621-TPS621.

Denkert C., von Minckwitz G., Darb-Esfahani S., Lederer B., Heppner B.I., Weber K.E., Budczies J., Huober J., Klauschen F., Furlanetto J., Schmitt W.D., Blohmer J.U., Karn T., Pfitzner B.M., Kümmel S., Engels K., Schneeweiss A., Hartmann A., Noske A., Fasching P.A., Jackisch C., van Mackelenbergh M., Sinn P., Schem C., Hanusch C., Untch M. and Loibl S. (2018). Tumour-infiltrating lymphocytes and prognosis in different subtypes of breast cancer: a pooled analysis of 3771 patients treated with neoadjuvant therapy. Lancet Oncol. 19, 40-50.

Derouane F., van Marcke C., Berlière M., Gerday A., Fellah L., Leconte I., Van Bockstal M.R., Galant C., Corbet C. and Duhoux F.P. (2022). Predictive biomarkers of response to neoadjuvant chemotherapy in breast cancer: Current and future perspectives for precision medicine. Cancers 14, 3876.

Elston C.W. and Ellis O. (1991). Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. Histopathology 19, 403-410.

Guiu S., Gauthier M., Coudert B., Bonnetain F., Favier L., Ladoire S., Tixier H., Guiu B., Penault-Llorca F., Ettore F., Fumoleau P. and Arnould L. (2010). Pathological complete response and survival according to the level of HER-2 amplification after trastuzumab-based neoadjuvant therapy for breast cancer. Br. J. Cancer 103, 1335-1342.

Guo S., Wang Y., Rohr J., Shang L. and Ma J. (2021). p63 expression is associated with high histological grade, aberrant p53 expression and TP53 mutation in HER2-positive breast carcinoma. J. Clin. Pathol. 74, 641-645.

Hammond M.E.H., Hayes D.F., Dowsett M., Allred D.C., Hagerty K.L., Badve S., Fitzgibbons P.L., Francis G., Goldstein N.S., Hayes M., Hicks D.G., Lester S., Love R., Mangu P.B., McShane L., Miller K., Osborne C.K., Paik S., Perlmutter J., Rhodes A., Sasano H., Schwartz J.N., Sweep F.C.G., Taube S., Torlakovic E. E., Valenstein P., Viale G., Visscher D., Wheeler T., Williams R.B., Wittliff J.L. and Wolff A.C. (2010). American Society of Clinical Oncology/College of American Pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer (Unabridged Version). Arch. Pathol. Lab. Med. 134, e48-e72.

Harvey J.M., Clark G.M., Osborne C.K. and Allred D.C. (1999). Estrogen receptor status by immunohistochemistry is superior to the ligand-binding assay for predicting response to adjuvant endocrine therapy in breast cancer. J. Clin. Oncol. 17, 1474-1481.

Ignatiadis M., van den Eynden G., Roberto S., Fornili M., Bareche Y., Desmedt C., Rothé F., Maetens M., Venet D., Holgado E., McNally V., Kiermaier A., Savage H.M., Wilson T.R., Cortes J., Schneeweiss A., Willard-Gallo K., Biganzoli E. and Sotiriou C. (2019). Tumor-Infiltrating lymphocytes in patients receiving Trastuzumab/Pertuzumab-Based chemotherapy: A TRYPHAENA Substudy. J. Natl. Cancer Inst. 111, 69-77.

Katayama A., Miligy I.M., Shiino S., Toss M.S., Eldib K., Kurozumi S., Quinn C.M., Badr N., Murray C., Provenzano E., Callagy G., Martyn C., Millican-Slater R., Purdie C., Purnell D., Pinder S.E., Oyama T., Shaaban A.M., Ellis I. Lee A.H.S. and Rakha E.A. (2021). Predictors of pathological complete response to neoadjuvant treatment and changes to post-neoadjuvant HER2 status in HER2-positive invasive breast cancer. Mod. Pathol. 34, 1271-1281.

Katayama A., Starczynski J., Toss M.S., Shaaban A.M., Provenzano E., Quinn C.M., Callagy G., Purdie C.A., Millican-Slater R., Purnell D., Chagla L., Oyama T., Pinder S.E., Chan S., Ellis, I. Lee A.H.S. and Rakha E.A. (2022). The frequency and clinical significance of centromere enumeration probe 17 alterations in human epidermal growth factor receptor 2 immunohistochemistry-equivocal invasive breast cancer. Histopathology 81, 511-519.

Kilmartin D., O'loughlin M., Andreu X., Bagó-Horváth Z., Bianchi S., Chmielik E., Cserni G., Figueiredo P., Floris G., Foschini M.P., Kovács A., Stovgaard E.S., Quinn C., Zolota V., Webber M., Roshan D., Glynn S. A., Callagy G. and Otero M.J. (2021). Intra-Tumour heterogeneity is one of the main sources of inter-observer variation in scoring stromal tumour infiltrating lymphocytes in triple negative breast cancer. Cancers 13, 4410.

Köbel M., Hoang L.N., Tessier-Cloutier B., Meng B., Soslow R.A., Stewart C. J.R. and Lee C.H. (2018). Undifferentiated endometrial carcinomas show frequent loss of core Switch/Sucrose nonfermentable complex proteins. Am. J. Surg. Pathol. 42, 76-83.

Kogawa T., Fouad T.M., Liu D.D., Wu J., Shen Y., Masuda H., Fujii T., Chavez-MacGregor M., Alvarez R.H., Hortobágyi G.N., Valero V. and Ueno N.T. (2016). High HER2/Centromeric probe for chromosome 17 fluorescence in situ hybridization ratio predicts pathologic complete response and survival outcome in patients receiving neoadjuvant systemic therapy with trastuzumab for HER2-Overexpressing locally advanced breast cancer. Oncologist 21, 21-27.

Korde L.A., Somerfield M.R., Carey L.A., Crews J.R., Denduluri N., Hwang E.S., Khan S.A., Loibl S., Morris E.A., Perez A., Regan M.M., Spears P.A., Sudheendra P.K., Symmans W.F., Yung R.L., Harvey B.E. and Hershman D.L. (2021). Neoadjuvant chemotherapy, endocrine therapy, and targeted therapy for breast cancer: ASCO guideline. J. Clin. Oncol. 39, 1485-1505.

Krystel-Whittemore M., Xu J., Brogi E., Ventura K., Patil S., Ross D.S., Dang C., Robson M., Norton L., Morrow M. and Wen H.Y. (2019). Pathologic complete response rate according to HER2 detection methods in HER2-positive breast cancer treated with neoadjuvant systemic therapy. Breast Cancer Res. Treat. 177, 61-66.

Lambein K., Praet M., Forsyth R., van den Broecke R., Braems G., Matthys B., Cocquyt V., Denys H., Pauwels P. and Libbrecht L. (2011). Relationship between pathological features, HER2 protein expression and HER2 and CEP17 copy number in breast cancer: Biological and methodological considerations. J. Clin. Pathol. 64, 200-207.

Lee H. J., Seo A.N., Kim E.J., Jang M.H., Kim Y.J., Kim J.H., Kim S.W., Ryu H.S., Park I.A., Im S.A., Gong G., Jung K.H., Kim H.J. and Park S.Y. (2015). Prognostic and predictive values of EGFR overexpression and EGFR copy number alteration in HER2-positive breast cancer. Br. J. Cancer 112, 103-111.

Li K., Liao N., Chen B., Zhang G., Wang Y., Guo L., Wei G., Jia M., Wen L., Ren C., Cao L., Mok H., Li C., Lin J., Chen X., Zhang Z., Hou T., Li M., Liu J., Balch C.M. and Liao N. (2020). Genetic mutation profile of Chinese HER2-positive breast cancers and genetic predictors of responses to Neoadjuvant anti-HER2 therapy. Breast Cancer Res. Treat. 183, 321-332.

Loibl S., Jackisch C., Schneeweiss A., Schmatloch S., Aktas B., Denkert C., Wiebringhaus H., Kümmel S., Warm M., Paepke S., Just M., Hanusch C., Hackmann J., Blohmer J.U., Clemens M., Costa S.D., Gerber B., Engels K., Nekljudova V., von Minckwitz G., Untch M. and Investigators of the German Breast Group (GBG) and the Arbeitsgemeinschaft Gynäkologische Onkologie-Breast (AGO-B) study groups (2017). Dual HER2-blockade with pertuzumab and trastuzumab in HER2-positive early breast cancer: a subanalysis of data from the randomized phase III GeparSepto trial. Ann. Oncol. 28, 497-504.

Lovell D.P. (2013). Biological importance and statistical significance. J. Agric. Food Chem. 61, 8340-8348.

Mercogliano M.F., de Martino M., Venturutti L., Rivas M.A., Proietti C.J., Inurrigarro G., Frahm I., Allemand D.H., Deza E.G., Ares S., Gercovich F.G., Guzmán P., Roa J.C., Elizalde P.V. and Schillaci R. (2017). TNFα-induced mucin 4 expression elicits trastuzumab resistance in HER2-positive breast cancer. Clin. Cancer Res. 23, 636-648.

Mougalian S.S., Hernandez M., Lei X., Lynch S., Henry M.K., Symmans W.F., Theriault R.L., Fornage B.D., Hsu L., Buchholz T.A., Sahin A.A., Hunt K.K., Yang W.T., Hortobagyi G.N. and Valero V. (2016). Ten year outcomes of breast cancer patients with cytologically confirmed axillary lymph node metastases and pathologic complete response after primary systemic chemotherapy. JAMA Oncol. 2, 508-516.

Nielsen T.O., Leung S.C.Y., Rimm D.L., Dodson A., Acs B., Badve S., Denkert C., Ellis M.J., Fineberg S., Flowers M., Kreipe H.H., Laenkholm A.V., Pan H., Penault-Llorca F.M., Polley M.Y., Salgado R., Smith I.E., Sugie T., Bartlett J.M.S., McShane L.M., Dowsett M. and Hayes D.F. (2021). Assessment of Ki67 in breast cancer: Updated recommendations from the international Ki67 in breast cancer working group. J. Natl. Cancer Inst. 113, 808-819.

O'Loughlin M., Andreu X., Bianchi S., Chemielik E., Cordoba A., Cserni G., Figueiredo P., Floris G., Foschini M.P., Heikkilä P., Kulka J., Liepniece-Karele I., Regitnig P., Reiner A., Ryska A., Sapino A., Shalaby A., Stovgaard E.S., Quinn C., Walsh E.M., Zolota V., Glynn S.A. and Callagy, G. (2018). Reproducibility and predictive value of scoring stromal tumour infiltrating lymphocytes in triple-negative breast cancer: a multi-institutional study. Breast Cancer Res. Treat. 171, 1-9.

Page P. (2014). Beyond statistical significance: clinical interpretation of rehabilitation research literature. Int. J. Sports Phys. Ther. 9, 726-736.

Salgado R., Denkert C., Demaria S., Sirtaine N., Klauschen F., Pruneri G., Wienert S., van den Eynden G., Baehner F.L., Penault-Llorca F., Perez E.A., Thompson E.A., Symmans W.F., Richardson A.L., Brock J., Criscitiello C., Bailey H., Ignatiadis M., Floris G., Sparano J., Kos Z., Nielsen T., Rimm D.L., Allison K.H., Reis-Filho J.S., Loibl S., Sotiriou C., Viale G., Badve S., Adams S., Willard-Gallo K. and Loi S. (2015). The evaluation of tumor-infiltrating lymphocytes (TILS) in breast cancer: Recommendations by an International TILS Working Group 2014. Ann. Oncol. 26, 259-271.

Sauter G., Lee J., Bartlett J.M.S., Slamon D.J. and Press M.F. (2009). Guidelines for human epidermal growth factor receptor 2 testing: Biologic and methodologic considerations. J. Clin. Oncol. 27, 1323-1333.

Singer C.F., Tan Y.Y., Fitzal F., Steger G.G., Egle D., Reiner A., Rudas M., Moinfar F., Gruber C., Petru E., Bartsch R., Tendl K.A., Fuchs D., Seifert M., Exner R., Balic M., Bago-Horvath Z., Filipits M. and Gnant M. (2017). Pathological complete response to neoadjuvant trastuzumab is dependent on HER2/CEP17 ratio in HER2-amplified early breast cancer. Clin. Cancer Res. 23, 3676-3683.

Solinas C., Ceppi M., Lambertini M., Scartozzi M., Buisseret L., Garaud S., Fumagalli D., de Azambuja E., Salgado R., Sotiriou C., Willard-Gallo K. and Ignatiadis M. (2017). Tumor-infiltrating lymphocytes in patients with HER2-positive breast cancer treated with neoadjuvant chemotherapy plus trastuzumab, lapatinib or their combination: A meta-analysis of randomized controlled trials. Cancer Treat. Rev. 57, 8-15.

Symmans W.F., Peintinger F., Hatzis C., Rajan R., Kuerer H., Valero V., Assad L., Poniecka A., Hennessy B., Green M., Buzdar A.U., Singletary S.E., Hortobagyi G.N. and Pusztai L. (2007).

Measurement of residual breast cancer burden to predict survival after neoadjuvant chemotherapy. J. Clin. Oncol. 25, 4414-4422.

Van Bockstal M.R., François A., Altinay S., Arnould L., Balkenhol M., Broeckx G., Burguès O., Colpaert C., Dedeurwaerdere F., Dessauvagie B., Duwel V., Floris G., Fox S., Gerosa C., Hastir D., Jaffer S., Kurpershoek E., Lacroix-Triki M., Laka A., Lambein K., MacGrogan G.M., Marchió C., Martin Martinez M.D., Nofech-Mozes S., Peeters D., Ravarino A., Reisenbichler E., Resetkova E., Sanati S., Schelfhout A.M., Schelfhout V., Shaaban A., Sinke R., Stanciu-Pop C.M., van Deurzen C.H.M., Van de Vijver K.K., Van Rompuy A.S., Vincent-Salomon A., Wen H.Y., Wong S., Bouzin C. and Galant C. (2021). Interobserver variability in the assessment of stromal tumor-infiltrating lymphocytes (sTILs) in triple-negative invasive breast carcinoma influences the association with pathological complete response: the IVITA study. Mod. Pathol. 34, 2130-2140.

Van Bockstal M.R., Noel F., Guiot Y., Duhoux F.P., Mazzeo F., van Marcke C., Fellah L., Ledoux B., Berlière M. and Galant C. (2020). Predictive markers for pathological complete response after neo-adjuvant chemotherapy in triple-negative breast cancer. Ann. Diagn. Pathol. 49, 151634.

von Minckwitz G., Huang C.-S., Mano M.S., Loibl S., Mamounas E.P., Untch M., Wolmark N., Rastogi P., Schneeweiss A., Redondo A., Fischer H.H., Jacot W., Conlin A.K., Arce-Salinas C., Wapnir I.L., Jackisch C., DiGiovanna M.P., Fasching P.A., Crown J.P., Wülfing P., Shao Z., Caremoli E.R., Wu H., Lam L.H., Tesarowski D., Smitt M., Douthwaite H., Singel S.M. and Geyer C.E. (2019). Trastuzumab emtansine for residual invasive HER2-Positive breast cancer. N. Engl. J. Med. 380, 617-628.

Waks A.G., Desai N.V., Li T., Poorvu P.D., Partridge A.H., Sinclair N., Spring L.M., Faggen M., Constantine M., Metzger O., Alberti J., Deane J., Rosenberg S.M., Frank E., Tolaney S.M., Krop I.E., Tung N.M., Tayob N., King T.A., Mittendorf E.A. and Winer E.P. (2022). A prospective trial of treatment de-escalation following neoadjuvant paclitaxel/trastuzumab/pertuzumab in HER2-positive breast cancer. NPJ Breast Cancer 8, 63.

Wolff A.C., Hammond M.E.H., Allison K.H., Harvey B.E., Mangu P.B., Bartlett J.M.S., Bilous M., Ellis I.O., Fitzgibbons P., Hanna W., Jenkins R.B., Press M.F., Spears P.A., Vance G.H., Viale G., McShane L.M. and Dowsett M. (2018a). Human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists Clinical Practice Guideline Focused Update. J. Clin. Oncol. 36, 2105-2122.

Wolff A.C., Hammond M.E.H., Hicks D.G., Dowsett M., McShane L.M., Allison K.H., Allred D.C., Bartlett J.M.S., Bilous M., Fitzgibbons P., Hanna W., Jenkins R.B., Mangu P.B., Paik S., Perez E.A., Press M.F., Spears P.A., Vance G.H., Viale G. and Hayes D.F. (2013). Recommendations for human epidermal growth factor receptor 2 testing in breast. J. Clin. Oncol. 31, 3997-4013.

Wolff A.C., McShane L.M., Hammond M.E.H., Allison K.H., Fitzgibbons P., Press M.F., Harvey B.E., Mangu P.B., Bartlett J.M.S., Hanna W., Bilous M., Ellis I.O., Dowsett M., Jenkins R.B., Spears P.A., Vance G.H. and Viale G. (2018b). Human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists Clinical practice guideline focused update. Arch. Pathol. Lab. Med. 142, 1364-1382.

Yau C., Osdoit M., van der Noordaa M., Shad S., Wei J., de Croze D., Hamy A.S., Laé M., Reyal F., Sonke G. S., Steenbruggen T.G., van Seijen M., Wesseling J., Martín M., del Monte-Millán M., López-Tarruella S., I-SPY 2 Trial Consortium; Boughey J.C., Goetz M.P., Hoskin T., Gould R., Valero V., Edge S.B., Abraham J.E., Bartlett J.M.S., Caldas C., Dunn J., Earl H., Hayward L., Hiller L., Provenzano E., Sammut S.J., Thomas J.S., Cameron D., Graham A., Hall P., Mackintosh L., Fan F., Godwin A.K., Schwensen K., Sharma P., DeMichele A.M., Cole K., Pusztai L., Kim M.O., van 't Veer L.J., Esserman L.J. and Symmans W.F. (2022). Residual cancer burden after neoadjuvant chemotherapy and long-term survival outcomes in breast cancer: a multicentre pooled analysis of 5161 patients. Lancet Oncol. 23, 149-160.

Zhao J., Krishnamurti U., Zhang C., Meisel J., Wei Z., Suo A., Aneja R., Li Z. and Li X. (2020). HER2 immunohistochemistry staining positivity is strongly predictive of tumor response to neoadjuvant chemotherapy in HER2 positive breast cancer. Pathol. Res. Pract. 216, 153155.

Accepted May 10, 2023