Original paper

Association of breast cancer grade with response to neoadjuvant chemotherapy assessed postoperatively

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Currently, breast cancer chemotherapy response can be predicted based on various parameters, with common reporting of tumour grade and Ki67 proliferation index. We analysed their association with pathological complete response (pCR) in a multivariate approach.

The study was carried out in a group of 353 patients, treated by preoperative chemotherapy and prospectively observed. In selected patients, parallel to routing core needle biopsy assessment, gene expression profile of tumour was analysed by oligonucleotide microarrays.

Tumour parameters associated with pCR in univariate analysis were: tumour grade, nuclear grade, mitotic index, Ki67, oestrogen and progesterone receptor (all $p < 0.0001$), and triple-negative status ($p = 0.0032$). The highest increase of pCR chance was observed in patients with high-grade tumours and with Ki67 ≥ 20%. In multivariate analysis, only tumour grade and oestrogen receptor status were predictive for pCR independently of other variables, with high grade increasing the odds of pCR 2.42 fold, and high ER decreasing the chance of pCR 0.41 fold. Tumour grading reflects important biological features of breast cancer and is not inferior to proliferation markers, including Ki67. It should be taken into account in decision-making for preoperative chemotherapy in parallel to breast cancer biologic subtypes, because grade 3 tumours exhibit a higher proportion of pCR.

Key words: breast cancer, preoperative chemotherapy, grading.

Introduction

The rising incidence of breast cancer and expanding portfolio of novel treatment options for this disease, confronted with limited funding, introduce a growing number of therapeutic dilemmas. Thus, breast cancer prognosis prediction becomes an increasingly important task in modern oncology, especially in the context of preoperative chemotherapy. The indications for preoperative use of systemic treatment in breast cancer are expanding, with a rising number of potential benefits of therapy administered in this setting. From breast conservation and lymph node sparing to post-operative maintenance systemic therapy in non-responding individuals, patients might both de-escalate the extent of therapy in the scenario of excellent response, and increase the treatment intensity in case of resistance. However, proper pre-treatment assessment of disease burden and aggressiveness is not straightforward, and no consensus on the optimal set of parameters exists [1, 2, 3, 4].

Current European guidelines mainly are based on the opinions of experts, as reflected in St. Gallen 2017 [5] guidelines and discussions during the 2019 meeting in Vienna. Subtyping of breast carcinomas into five subgroups based on immunohistochemical assessment of oestrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor type 2 (HER2), and Ki-67 proliferation index. Both PR and Ki-67 are used to discriminate between the 'Luminal A-like' and 'Luminal B-like (HER2-negative)' subtypes, with a clear impact on predicted outcome and chemotherapy indications.

The eighth edition of the American Joint Committee on Cancer (AJCC) cancer staging manual introduced information about tumour biological parameters into the staging system, namely tumour grade, ER/PR/HER2 status and – for post-operative assessment only – the result of molecular tumour testing by OncotypeDx 21-gene panel [6]. Thus, the position of tumour grade in this system is central, similar to the importance given in European guidelines to Ki67 proliferation index. As both indices are prone to error when assessed in relatively small specimens, taken by core needle biopsy preoperatively, their use to predict the outcome of preoperative chemotherapy is more challenging than the application in the post-operative setting. However, the extending indications for preoperative chemotherapy in breast cancer make the issue of preoperative chemosensitivity prediction one of most important diagnostic goals in this disease. It is also relevant nowadays for luminal HER2-negative tumours, which may influence both breast conservation rate and reduce the volume of surgery in axilla.

As the application of tumour grade to predict chemotherapy response was recently overtaken by Ki67 proliferation score, and the mutual application of both parameters is a matter of debate, in this study we analysed the association of tumour grade with pathological complete response after preoperative chemotherapy, in context of other important tumour parameters including proliferation kinetics.

Material and methods

We analysed the data of 353 patients with breast cancer, all females, with mean age 46.5 years (95% confidence interval 45.4-47.7 years, median 44.6 years). Patients were enrolled prospectively after giving informed consent, before preoperative chemotherapy, either due to locally advanced or oligometastatic breast cancer or early-stage disease, with intent of breast-conserving surgery after neoadjuvant chemotherapy. The study was approved by the local Ethics Committee. Within the group there were 208 (60.3%) patients with tumour size T1-T2, 66 (19.1%) T3 patients, and 71 (20.6%) females with advanced T4 tumour. No nodal metastases were found in 122 patients (cN0, 35.6%), 117 (34.1%) patients had N1 nodal involvement, 72 (21.0%) fixed/matted N2 lymph node metastases and 32 (9.3%) advanced (N3) lymph node involvement (for 2.3% and 2.8% of patients no T or N stage was defined, respectively – Tx or Nx). Patients were subjected to initial chemotherapy; the majority of patients (77.4%) received anthracycline and taxane chemotherapy (137 (41.6%) received docetaxel/doxorubicin or epirubicin/cyclophosphamide – TAC/TEC regimen; 132 (40.1%) sequential doxorubicin/cyclophosphamide-paclitaxel AC-P regimen), and 60 (18.2%) received anthracycline-only regimen (5-fluorouracil/doxorubicin/ cyclophosphamide, FAC). In 6.8% of the whole group other drug combinations were used (docetaxel-doxorubicin, docetaxel-cyclophosphamide, paclitaxel). 197 (55.8%) patients were hormone-sensitive and HER2-negative, 55 (15.6%) hormone-sensitive and HER2-positive, 34 (9.6%) were non-luminal HER-positive, and 67 (19.0%) had triple-negative breast cancer. In total, 241 (68.5%) of the whole group showed hormone responsiveness (positive oestrogen or progesterone staining on immunohistochemical staining, at least 1%) and in 89 (25.7%) patients positive HER2 status was confirmed (either +++ in immunohistochemical staining or positive FISH in patients with ++ status).

Among the patients there were 38 (10.9%) individuals with metastatic disease (M1) at initial presentation, and 53 patients (15%) were not treated surgically after initial chemotherapy (no sufficient disease response). 300 patients reached surgery, and the pathological complete response on postoperative examination was defined as the disappearance of invasive tumour both from the tumour bed in breast and axillary lymph nodes. 77 patients (25.7%) showed pathological complete response (pCR), while in 223 (74.3%) individuals there was no pCR.

In 72 patients, a second core needle biopsy was taken, after the initial histopathological assessment (the main reason for second biopsy was no clip insertion to mark the tumour bed in the patient before preoperative chemotherapy). We assessed the concordance of grading and Ki67 assessment between first and second biopsy (the second study was assessed by one of two histopathologists involved, E.S. or E.C.).

From 25 patients from the analysed group during pre-treatment core needle biopsy, upon the acceptance of Local Ethics Committee and after the patients' informed consent, additional tissue material was collected and stored in RNAlater. Total RNA was obtained by homogenisation of frozen tissue using a Tissuelyser II (Qiagen GmbH, Hilden, Germany) followed by extraction and purification using RNeasy Mini Kits (Qiagen). RNA quality was estimated by Agilent 2100 using RNA 6000 Nano Assay (Agilent

Table I. Association of selected tumour parameters with pathological complete response, as assessed postoperatively. Chi-square test or Fisher's exact test (2 × 2 tables) are given; $p < 0.05$ was deemed significant and is marked by an asterisk

Variable		pCR	No pCR or no surgery	Total	P-value
		Count Row %			
Grade	G1	2 6.67%	28 93.33%	30	< 0.0001*
	G2	10 8.55%	107 91.45%	117	
	G3	61 31.28%	134 68.72%	195	
	Gx	4 36.36%	7 63.64%	11	
	Total	77	276	353	
Nuclear Grade	NG1	1 7.14%	13 92.86%	14	< 0.0001*
	NG2	10 8.33%	110 91.67%	120	
	NG3	64 30.92%	143 69.08%	207	
	NGx	2 40.00%	3 60.00%	5	
	Total	77	269	346	
Mitotic index	≤ 20	31 15.74%	166 84.26%	197	0.0016*
	> 20	40 30.53%	91 69.47%	131	
	Total	71	257	328	
Ki67	< 20%	4 6.15%	61 93.85%	65	< 0.0001*
	≥ 20%	72 26.57%	199 73.43%	271	
	Total	76	260	336	
ER	Negative or low	54 36.24%	95 63.76%	149	< 0.0001*
	High	23 11.33%	180 88.67%	203	
	Total	77	275	352	
PR	Negative or low	60 30.77%	135 69.23%	195	< 0.0001*
	High	17 10.83%	140 89.17%	157	
	Total	77	275	352	
HER2	Positive	26 29.21%	63 70.79%	89	0.0584
	Negative	50 19.38%	208 80.62%	258	
	Total	76	271	347	
TNBC (negative ER. PR and HER2)	TNBC	24 35.82%	43 64.18%	67	0.0032*
	Other	53 18.53%	233 81.47%	286	
	Total	77	276	353	

Table II. Clinical and laboratory characteristics of prostate cancer in G84E carriers and non-carriers

Variable	Levels compared	LogWorth	P value	Odds Ratio	Confidence Interval	
					Lower 95%	Upper 95%
Grade	(G3 or Gx) vs. (G1-2)	1.751	0.0178*	2.42	1.16	5.33
ER	High vs. low/moderate	1.323	0.0475*	0.41	0.16	0.99
Ki67	≥ 20% vs. < 20%	0.974	0.1063	2.41	0.84	8.69
PR	Negative or low vs. high	0.314	0.4857	1.38	0.54	3.39
TNBC	TNBC vs. other	0.215	0.6098	0.80	0.33	1.90
HER2	Positive vs. negative	0.001	0.9982	1.00	0.47	2.09

Technologies, Santa Clara, CA, United States). RNA integrity was assessed by RNA Integrity Number (RIN) index (Agilent). RNA quantity was measured by NanoDrop ND-1000 minispectrophotometer.

In all 25 patients 3' oligonucleotide gene expression analysis was carried out by Affymetrix HG-U133Plus2 microarrays. The analysis was performed according to the recommendations of the Affymetrix Gene Expression Analysis Technical Manual (Santa Clara, CA, United States). Briefly, 2 μg of total RNA was used as a template for cDNA synthesis (One-Cycle cDNA Synthesis Kit, Affymetrix), and a further in vitro transcription step was performed using an IVT Labeling Kit (Affymetrix). Labelled cRNA was purified by GeneChip Sample Cleanup Module, and the quality of biotinylated cRNA was evaluated by capillary electrophoresis (Bioanalyzer 2100, Agilent) and then fragmented and hybridised to Human Genome U133 2. Plus 2.0 array (Affymetrix). After washing and staining with streptavidin-phycoerythrin conjugate the arrays were scanned in a GeneChip 3000G scanner (Affymetrix).

Table III. Comparison of tumour grade as reported in primary histopathological report to the second-read re-analysis by two experienced pathologists

		Re-analysis of tumour grade			
		G1	G2	G3	Total
Initial tumour grade	G1	7 87.50%	0	1 12.50%	8
	G2	2 6.67%	21 70.00%	7 23.33%	30
	G3	0	2 6.25%	30 93.75%	32
	Gx	0	1 50.00%	1 50.00%	2
	Total	9	24	39	72

Results

Univariate association of histological parameters with pCR

Association of various tumour parameters on initial core needle biopsy with response to chemotherapy was analysed. Patients with pathological complete response (pCR) were compared to individuals without pCR in postoperative histopathological examination or to patients who did not exhibit clinical response sufficient to undergo surgery (no pCR/no surgery group).

Tumour grade was clearly associated with pCR status in univariate analysis. Among patients with G1 tumours (30 pts) or G2 tumours (117 patients) pCR was relatively rare, occurring in 6.7% and 8.6% of patients, respectively. Conversely, in G3 patients approx. one third (31.3%) showed pathological complete response ($p < 0.0001$). Of note, a relatively large proportion of patients in whom no grading was provided in core needle assessment (Gx) showed also pCR (4 out of 11 patients, 36%).

A similar association strength as for the overall tumour grade was observed also for the Nuclear Grade subscore. Patients with NG1 and NG2 tumours showed relatively low rate of pCR (7.1% and 8.3%, respectively), while in patients with high nuclear grade (NG3) there were 30.9% of pCRs ($p < 0.0001$). Again, NGx patients showed high pCR rate (40%). Less pronounced differences were observed in the context of tumour mitotic index. Patients with less than 20 mitoses /10 HPF showed average pCR rate (15.7%), while in tumours with high mitotic index (> 20 mitoses / 10 HPF) the pCR rate was 30.5% ($p = 0.0016$).

Ki67 staining differentiated subgroups with high statistical significance ($p < 0.0001$), although the rate of pCR in patients with Ki67 > 20% was lower than in tumours graded G3 or with high mitotic index – 26.6% of patients in this subgroup showed pCR compared to 6.2% in patients with Ki67 ≤ 20%.

Higher pCR rate was also observed in patients with negative or low oestrogen or progesterone receptors. Negative/low ER resulted in pCR in 36.2% of patients, while for PR pCR was observed in 30.8%, compared to only 11.3% and 10.8% of patients with pCR in the ER high and PR high groups, respectively (both differences highly significant, $p < 0.0001$). In contrast, HER2-positive patients (as defined by IHC with additional FISH in ambiguous cases) did not clearly show any significant difference, although a trend for higher pCR rate was noted (29.2% vs. 19.4%, $p = 0.06$). It should be highlighted that during the study period the patients did not receive any preoperative HER2-targeted therapy (not reimbursed in Poland at that time). A greater magnitude of differences was observed in patients with negative staining for ER, PR, and HER2 (triple-negative cancers, TNBC); in this subgroup pCR was observed in 35.6% of patients compared to 18.5% in non-TNBC subjects ($p = 0.0032$).

Discriminatory univariate power of parameters to predict pCR

The single marker potential of parameters to predict pCR was assessed by odds ratio (OR). The increase in probability of pCR was 5.2-fold both for tumour grade (G3/Gx vs. G1/G2) and for Ki67 (≥ 20% vs. < 20%) (OR = 5.186, 95% CI: 2.682-10.029). A slightly lower difference was noted for ER status "negative"/"low" (vs. "high") – the odds ratio for pCR was 4.448 (95% CI: 2.572-7.692); the same statistic for progesterone receptor status PR was 3.66 (95% CI: 2.033-6.591). The diagnosis of triple-negative cancer resulted in moderate OR = 2.454 (95% CI: 1.372-4.39); the increase of pCR probability associated with HER2-positive subtype was clearly lowest (OR = 1.717, 95% CI: 0.989-2.98).

Multivariate analysis

Important parameters (tumour grade, Ki67, ER, PR, HER2 and negative status for all three markers – TNBC) were analysed in multivariate logistic regression model to predict pCR status. Nuclear grade and mitotic index, as they are strongly – by definition – correlated with overall tumour grade, were not included. Two parameters – tumour grade and oestrogen receptor status, were predictive for pCR and independent from other analysed variables. Tumour grade was clearly significant ($p = 0.02$), with estimated odds ratio 2.42 (95% CI: 1.16-5.33), while for ER status the chance of pCR associated with high expression was 0.41 (95% CI: 0.16-0.99), with p value slightly below the significance limit ($p = 0.048$). The magnitude of effect estimated for Ki67 was similar to the tumour grade, but in the context of other factors Ki67 was not significant; the same was true for PR status but with much smaller estimated odds ratio. HER2 status in the context of other parameters was totally insignificant, while TNBC, although not statistically significant, carried a poor prognostic message similarly to the one observed in clinical practice.

The predictive power of the model was assessed by receiver operating characteristic curve (ROC). Area under curve (AUC) was estimated at 0.733, and the model R^2 was 0.121, indicating relatively moderate predictive power of all analysed parameters (Fig. 1).

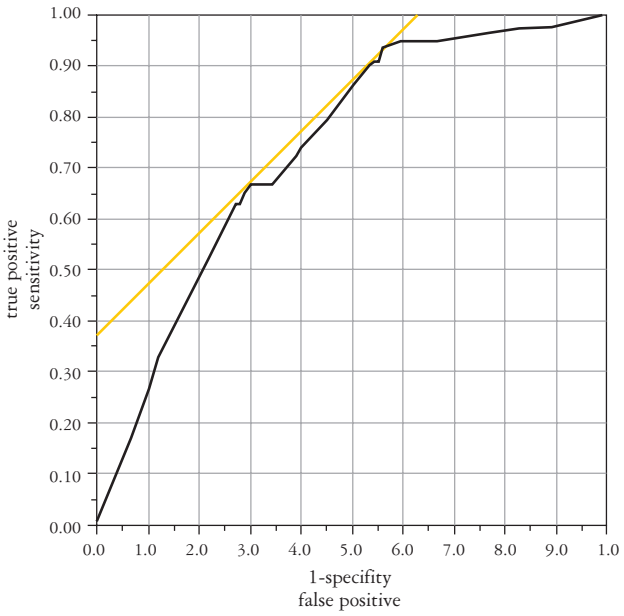

Fig. 1. Receiver Operating Characteristic (ROC) analysis of sensitivity versus specificity of multivariate model analysed in the study, based on tumour grade, oestrogen receptor expression, and other parameters of low significance. Area under curve (AUC) was estimated at 0.733, and the model R^2 was 0.121, indicating relatively moderate predictive power of all analysed parameters

Re-assessment of tumour grade and Ki67

For a subgroup of samples, we carried out a separate (additional) core needle biopsy. We compared tumour grade based on the initial biopsy to the re-assessment in a larger number of cores. In the majority of G3 tumours (93.8%) and G1 tumours (87.5%) there was an agreement of initial and additional grading assessment. Only single specimens showed discordance (G1-G3 in one case and G3-G2 in two cases). However, a greater proportion of discordances was observed in samples initially assessed as G2. In these tumours, 23.3% were assessed as G3 in second biopsy and in 6.7% as G1. In total, eight samples showed higher grade in second biopsy than in the first one, while in four tumours the grade was lower than the initial one. Two Gx tumours appeared as G2 and G3 in the second biopsy. The overall κ statistic indicating concordance of both observations was 0.68 (95% CI: 0.53-0.83).

The assessment of concordance in Ki67 analysis between initial and additional biopsy showed relatively good correlation, with R^2 of the model 0.71. The concordance was higher among low and high values, and within the moderate Ki67 range we observed the largest variability (Fig. 2).

Re-analysed tumour grades and Ki67 values were associated with similar pCR rates as the initial values (detailed analysis not shown).

Analysis of gene expression parameters

For a subset of samples, we analysed genomic data for transcripts representing major breast cancer features, as analysed in the study. They included ER (ESR1), PR (PGR), HER2 (ERBB2), Ki67 (MKI67), and two other transcripts representing proliferative potential of breast cancer (AURKA and CDK1). There was a clear association of ESR1 and PGR high expression with grade 1-2 tumours, with significant difference for ESR1 ($p = 0.01$) and trend for PGR ($p = 0.09$), although no full delineation of subgroups was possible. The remaining genes, especially all three transcripts associated with proliferation, did not show any clear association with tumour grade (Fig. 3).

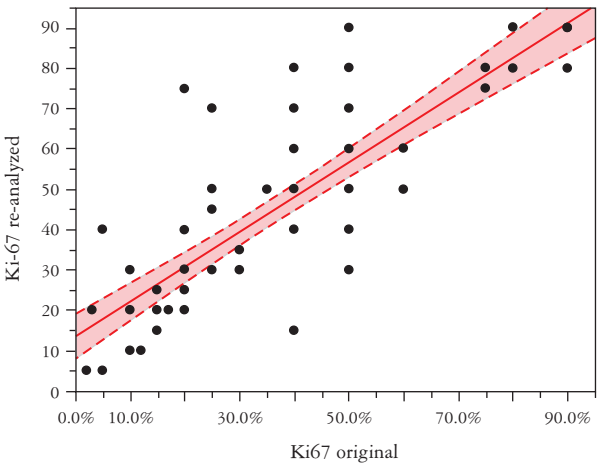

Fig. 2. Correlation between the assessment of tumour grade by the initial histopathological report and re-analysis by a second reviewer. Regression line is provided with 95% confidence intervals

Discussion

Our study was driven by the accumulating data suggesting the important role of tumour grade in breast cancer, supporting the tumour intrinsic subtypes, and widely considered as the most important driver of breast cancer biologic and clinical behaviour. It was motivated by a recently published Swedish study [7], analysing the role of tumour grade in the context of St Gallen surrogate definition of the intrinsic subtypes of breast cancer. The authors hypothesised that grade may be a primary feature to discriminate between ER-positive/HER2-negative breast cancers with good and bad prognosis, with Ki-67 and PR supporting discrimination in ambiguous grade 2 tumours. The authors analysed more than 600 patients with luminal tumours, confirming that the vast majority of Luminal A cancers are G1/G2, while the most common presentation of Luminal B cancer is G2/G3 stage. The authors also suggested that the grade could independently influence the prognosis in St Gallen subtypes, pointing out Luminal B tumours that were G1 in which no metastasis occurred and a subset (approx. one third) of Luminal A tumours that were G3 and metastasised. In the G2 subgroup PR and Ki67 helped discriminate between cancers with bad and good prognosis. The authors concluded that luminal G1 tumours exhibit good prognosis, while G3 tumours exhibit bad prognosis, and subtyping according to Ki67 and PR should be restricted to G2 specimens [7]. Because this suggestion contradicts our current practice (St Gallen surrogate intrinsic subtype is considered a more influential tumour feature than its grade), we undertook the analysis to compare the influence of tumour grade and immunohistochemical parameters.

We noted that in multivariate analysis tumour grade outperforms slightly proliferation-only-based Ki67 and is much more potent than the progesterone receptor itself. As the debate on the independent role of Ki67 has a long history, we do not extend this part of discussion here and direct the reader to numerous meta-analyses, reviews, and methodological papers [7, 8, 9, 10, 11, 12, 13]. Based on our data, we do not claim that tumour grade should be interpreted over Ki67 as a predictor of response and risk; we only stress the importance of grading as an ad-

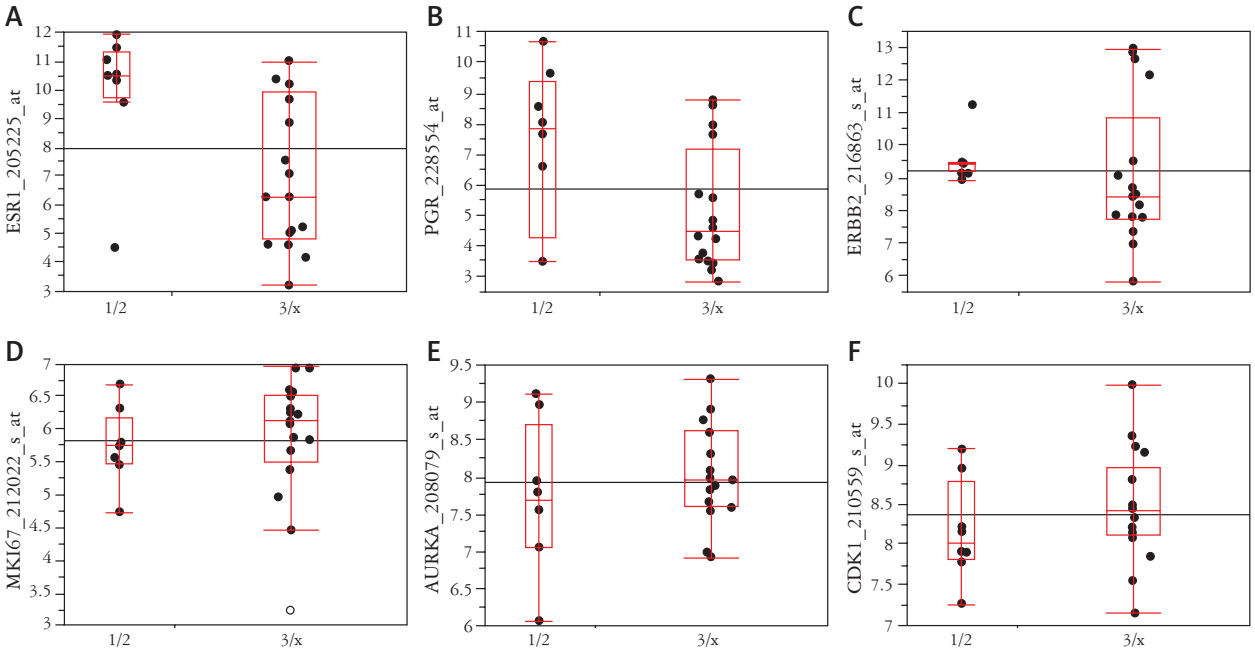

Fig. 3. Comparison of gene expression for selected transcripts representing important breast cancer markers (ESR1, PGR, ERBB2) or proliferation-related genes (MKI67, AURKA, CDK1). While for ESR1 a statistically significant difference was observed between grade 1/2 and grade 3 tumours ($p < 0.05$), for the remaining transcripts no significant differences were observed

junct method of prognosis determination. In patients with coherent grade and Ki67 results no modification is required in the decision-making process; the problem arises in patients with low grade and higher Ki67 scores or the opposite configuration of parameters. In these patients, the relatively high predictive power of tumour grade should be kept in mind. It is also important to stress that in a number of studies high Ki67 is associated with a high probability of pCR [14], with the latter leading to better overall prognosis; however, generally in studies addressing the population of patients in a neoadjuvant chemotherapy setting high initial Ki67 is related to poorer prognosis [12]. One of the reasons might be the fact that pCR rate in older neoadjuvant chemotherapy studies was relatively low (estimated to be 24% in meta-analysis by Criscitello *et al*. [15]) and increased mainly in the more aggressive subtypes by the occurrence of newer treatment regimens, mainly anti-HER2 therapy. A recent EBCTCG meta-analysis was ununable to extract proliferation indices, generally not reported in earlier trials. The overall pCR rate reported by EBCTCG is very similar (24.5%), with high-grade oestrogen receptor-negative tumours exhibiting greater chances of pCR, and low-grade ER-positive ones having decreased odds of complete response.

Ki67 was clearly associated with pCR rate in triple-negative breast cancer; in a meta-analysis it was estimated that high Ki67 is related to a 3.4-fold increase in pCR rate [16].

Lips *et al.* [17] carried a study demonstrating that breast cancer subtyping by immunohistochemistry and histological grade outperforms breast cancer surrogate intrinsic subtypes in predicting neoadjuvant chemotherapy response. In a subset of 560 patients, within the ER+/HER2- subgroup, a high histological grade was the best predictor for chemotherapy benefit, both in terms of pCR as well as progression-free survival time [17]. In this study, surrogate intrinsic subtypes based on Ki67 had no additional value over histological grade, ER, PR, and HER2.

Sotiriou *et al*., in the early days of studies on breast cancer genomic signatures, proposed to derive them from differences between grade 1 and grade 3 tumours [18, 19] and showed that a classifier derived that way allows reclassification of grade 2 tumours in a relatively robust manner. In fact, the group proposed that the majority of classification power in breast cancer is coming from proliferation genes, and they confirmed that finding by meta-analysis [20]. This was even further extended to claim that the proliferation gene content is so high that even randomly selected signatures carry predictive potential [21]. Although these finding await application in wider practice to aid discrimination of ambiguous grade 2 tumours, they also confirm the robustness of diagnosis of grade 1 and grade 3 tumours.

Still the grading of breast cancer, currently in the majority of centres by Elston-Ellis modification of the initial Scarf-Bloom-Richardson system (also called Nottingham breast cancer grade), has a suboptimal

interobserver agreement. However, results of grade assessment in breast cancer were shown to be highly correlated with at least 40 other morphological tumour features, and high-grade versus low-grade discrepancies were very rare [22]. Thus, grading still is the most common framework of assessment of breast cancer aggressiveness, clearly more widespread worldwide than Ki67.

Interesting results were reported in the WSG-AGO EC-Doc trial [15]. In this study, the authors compared clinical grading assessment and immunohistochemical parameters. It was found that genomic grade adds prognostic information to clinical grade assessment and tumour subtyping by surrogate definition of subtypes, while immunohistochemical assessment did not provide any additional value. The authors concluded that the high interobserver variability for histological grade and the still missing validation of Ki-67 preclude prescribing adjuvant chemotherapy based on these single factors alone.

Although disease progression during neoadjuvant breast cancer chemotherapy is relatively rare [23], the optimal selection of patients who may benefit from chemotherapy administered in a preoperative setting is crucial. The importance of proper selection increases with the growing number of treatment options for patients with breast cancer. The growing role of genomic approaches by complex or simpler predictors [24] is to be appreciated, but until they become more widespread in clinical practice, both tumour grade and surrogate St Gallen subtypes should be considered important features predicting the probability of pCR.

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