The effects of neoadjuvant chemotherapy on grade and proliferation of breast cancer

Ph. D. Thesis

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LIST OF PAPERS THAT SERVED AS THE BASIS OF THE PH.D. THESIS

I. **Ádám Ferenczi**, Gábor Cserni. Changes in breast cancer grade from biopsy to excision following surgery or primary chemotherapy. Pathologica 2024; 116:22-31, doi: 10.32074/1591-951x-958

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II. Tamás Zombori, **Ádám Ferenczi**, Anita Sejben, Szintia Almási, Veronika Szelestei, Renáta Koszó, Tamás Lantos, Zsuzsanna Kahán, Gábor Cserni. The prognostic value of histological grade determined after neoadjuvant chemotherapy of breast cancer. Pathol Res Pract 2025; 265:155732, doi: 10.1016/j.prp.2024.155732

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III. **Ádám Ferenczi**, Tamás Lantos, Gábor Cserni. Temporality of change in Ki67 labelling following neoadjuvant systemic chemotherapy of breast cancer – The common drop often subsides with time. Virchovs Arch 2025; Nov 13, doi: 10.1007/s00428-025-04263-7

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1. INTRODUCTION

Breast cancer is an epidemiologically important disease on the basis of both its prevalence and its mortality. The treatment of neoplastic disease is more and more shifted toward personalization. For this, many parameters need to be assessed, including prognosticators (variables with proven effect on outcome) and predictive markers (variables predicting the effectiveness of a given treatment). The pathology report contains many of these, allowing selection of the most appropriate treatment plan. Along with many other factors, breast cancer reports must contain the histological grade (G) according to the Nottingham grading system.

Histological grade reflects the differentiation and biological behaviour of the tumour, and in the case of breast cancer, it is determined by summing the subscores for tubule formation (T), nuclear pleomorphism (P) and area adjusted mitotic rate (M), each being scored on a three-tiered scale. According to the sum of these subscores, the tumour can be well differentiated (Grade 1, 3-5 points), moderately differentiated (Grade 2, 6-7 points) or poorly differentiated (Grade 3, 8-9 points). The evaluation of grade contains subjective factors and may be influenced by the experience of the evaluator, thus its reproducibility is considered moderate; nonetheless the prognostic significance of grade remains clear even in the era of modern molecular testing and gene expression profiling.

The treatment of breast cancer includes loco-regional modalities like surgery and radiotherapy, and systemic options acting at distant sites, but also locally and regionally, like endocrine therapy, chemotherapy, targeted treatments and immunotherapies.

In the era of molecular diagnostics, breast cancer has become a rather heterogenous disease. Its main gene expression-based molecular subtypes are luminal A, luminal B, basal-like and HER2-enriched. However, in everyday clinical practice, genomic tests are not routinely used, therefore, surrogate molecular subtypes have been defined as luminal A-like, triple negative (TN), HER2-positive and luminal B-like tumours, the latter being a quite heterogenous and not well-defined group.

Systemic chemotherapy for non-metastatic breast cancer may be administered before and/or after surgery and thus we can define it as neoadjuvant or adjuvant. According to several guidelines, histological grade must be assessed from both preoperative core needle biopsy (CNB) and surgical excision (EXC) specimens, as well as following neoadjuvant chemotherapy (NACT), although its prognostic value has less evidence in this latter scenario. NACT is more and more often administered, due to its ability to downstage locally advanced tumours, thus allowing for breast-conserving surgery and also serving as a way to monitor tumour response to treatment; it can also eliminate micrometastases or even greater metastatic deposits in high-risk early or locally advanced breast cancers.

Reporting the response of the tumour to NACT is also a core element of the pathology report according to the many recommendations. For this purpose, according to the most recent Hungarian guidelines, the Residual Cancer Burden (RCB) and European Working Group for Breast Screening Pathology (EWGBSP) Tumour Response (TR) and Nodal Response (NR) categories should be used while reporting. The RCB system takes into account the primary invasive tumour bed area, overall cancer cellularity, percentage of in situ disease, number of positive lymph nodes and diameter of the largest metastasis, resulting in RCB 0, -I, -II and -III categories. The EWGBSP TR system contains 3 major categories with further subdivision in two. The condition for reporting pathological complete response (pCR) is the complete processing of the tumour bed and all resected lymph nodes and their lack of any tumour cells; pCR has been found to be a good predictor of survival.

Most current systemic chemotherapy regimens for breast cancer contain taxanes (eg. docetaxel or paclitaxel) combined with anthracyclines (eg. doxorubicin or epirubicin) or platinum-based chemotherapeutic agents in case of cardiac comorbidities. The general effect of chemotherapy can be described as a reduction in the proliferating fraction of the tumour by inhibiting different components of the mitotic cycle.

Tumour proliferation can be assessed, among many other ways, by Ki67 immunohistochemistry (IHC) and the labelling index (Ki67 LI) which describes the percentage of staining cells. Ki67 is a nuclear protein expressed during the G1, S, G2 and M phases of the cell cycle, but not in the resting state, G0. Ki67 LI has been found to be an independent prognosticator for both overall survival (OS) and recurrence-free survival (RFS) when assessed from core biopsies (CNB-Ki67), postneoadjuvant surgical specimens (yKi67), as well as the change of these values from biopsy to excision specimen (ΔKi67). Prior research has also shown that high Ki67 LI is a strong predictor of pCR. Despite profound literature concerning Ki67 and its prognostic value, the dynamics and temporality of change in Ki67 LI following NACT are not well documented.

2. AIMS

1. To describe the changes observed in histological grade of breast cancer and its subscores for tubule formation, nuclear pleomorphism and mitotic rate following NACT and compare these with cases operated on without NACT.
2. To assess the prognostic significance of yG seen following NACT of breast cancers.
3. To analyse tumour proliferation as reflected by Ki67 IHC before and after NACT and evaluate its dynamics.

3. MATERIALS AND METHODS

The studies reported were granted ethical approval by the Regional and Institutional Review Board of Human Investigations in University of Szeged (91/2021 SZTE-RKEB).

3.1 ASSESSMENT OF CHANGES IN HISTOLOGICAL GRADE AND ITS COMPONENTS FOLLOWING NEOADJUVANT CHEMOTHERAPY

Data of patients with invasive breast cancer diagnosis from the time period of 2010 to 2022 were collected from the digital medical records of the Bács-Kiskun County Teaching Hospital. The patients were required to have both CNB and EXC specimens available.

Of all the cases seen at the Department of Pathology, the following were excluded from the study:

- multifocal tumours in which the histological grade of the foci was different, and it was unclear which focus was sampled preoperatively by the CNB (this exclusion was not applied to cases in which the multiple foci possessed identical histological grade);
- the CNB specimen was crushed or had limited diagnostic value, leading to the assessment of grade being inadequate (e.g., tumour dimension of less than 10 high power fields corresponding to 2 mm^2 or crush artefacts leading to uncertain assessment of nuclear pleomorphism or lumen formation);

- tumours with the diagnosis of in situ carcinoma from CNB;
- cases receiving neoadjuvant endocrine therapy;
- cases displaying pathological complete response or the residual disease being unsuitable for grading.

In all included cases, histological grade and its subscores were extracted from the original reports, and all data were recorded in Microsoft Excel spreadsheets. The cases were divided into groups according to the type of therapy received: the group receiving NACT was named PST, while the group treated with primary surgery was named CHIR. Histological grade was assessed according to the Nottingham grading system by a pathologist expert in breast pathology. For each case and its pair of samples before and after treatment, the three-tiered values of histological grade (G), T, P and M were recorded. The corresponding variables were matched for the CNB and EXC specimens of each case, thus representing the first and second measurement of that variable. The distributions for each value of every variable in the CHIR and PST groups, as well as the concordance rates in both groups were assessed by Chi-square test for both CNB and EXC specimens.

Assessment of change in all variables was carried out by nonparametric Wilcoxon signed rank test, as it accounts for negative and positive changes in the variables and analyses whether the change seen is random or carries some sort of tendency. The statistical calculations were carried out with the Microsoft Excel's Real Statistics Resource Pack add-in. The final, resulting z-score in a two-tailed test with 95% confidence intervals was significant above 1.96. Level of significance was $p < 0.05$.

3.2. EXAMINATION OF THE PROGNOSTIC VALUE OF POSTNEOADJUVANT GRADE

The retrospective study of consecutive cases included breast cancer patients who received NACT followed by surgery at the Departments of Oncotherapy and Surgery of the Albert Szent-Györgyi Clinical Centre of the University of Szeged between 1999 and 2018, as well as patients treated at the Bács-Kiskun County Teaching Hospital, Kecskemét, between 2000 and 2018.

Exclusion criteria in this study were the lack of sufficient residual tumour or undeterminable yG due to poor tissue fixation, as well as detection of distant metastasis (M1, stage IV) within 6 months from initial diagnosis, and lacking follow-up data from the digital charts.

For all included cases, multiple clinicopathological parameters, such as gender, age, histological diagnosis, histological grade from the biopsy specimen (BxG) of the tumour, type of NACT, type of surgery, completeness of resection, biopsy based ER, PR and HER2 status, presence or absence of lymphovascular invasion, yG, ypT, ypN categories, and lastly overall survival (OS) and recurrence free survival (RFS) were collected and recorded in Microsoft Excel spreadsheets. For survival assessment, time from initiation of systemic treatment to last follow-up or recurrence (RFS) / death (OS) was used; patients lost to follow-up were censored at the last follow-up date.

The change in histological grade has been assessed with contingency tables and Chi-square test in this cohort of cases, too. The prognostic value of yG was assessed using Kaplan-Meier analyses and log-rank test, and to further solidify our findings, univariate Cox proportional hazards model was applied as well. Each variable proving significant in the univariate model has also been tried in a multivariate model. The statistical calculations and creation of graphs were carried out using SPSS Statistics V 23.0 software (Armonk, USA).

3.3. DESCRIPTION OF THE CHANGE IN KI67 LABELLING INDEX FOLLOWING NEOADJUVANT TREATMENT AND ITS TEMPORALITY

Invasive breast cancer cases treated with NACT with both CNB and EXC specimens available were collected at the Bács-Kiskun County Teaching Hospital between 2009 and 2024. Inclusion required Ki67 immunohistochemistry (IHC) stained slides available for both samples, and cases with technically suboptimal staining, cut out material, and tumours only available within megaslides were excluded.

For each of the included cases, data such as patient age, tumour type, grade, ER, PR and HER2 status, Ki67 IHC results via eye-balling based subjective estimation of the percentage of stained tumour cells by an expert pathologist (EB-EST), T, P and M scores, mitotic index (MI: number of mitoses per 10 high power fields, corresponding to 2 mm^2), exact dates of surgery and last cycle of NACT prior to surgery, type of NACT and response to treatment were collected in Microsoft Excel spreadsheets. The tumour response was characterized according to RCB categories I, II and III and the EWGBSP TR categories 2a, 2b, 2c and 3, where the RCB-I and TR2a categories represent a good response to treatment (minimal residual disease), while RCB-III and TR3 categories represent no response at all. Apart from the relatively subjective EB-EST method, the proportion of Ki67 positive cells was also determined using 2 more objective methods.

The relevant Ki67 stained slides were retrieved from the archives of the Department and scanned for each case using a Pannoramic 250 scanner (3DHistech, Budapest, Hungary). Three images were taken from each slide with the SlideViewer software (3DHistech, Budapest, Hungary) at 30X magnification; these pictures served as the basis of our further analyses.

One method involved the ImmunoRatio (IR) 1.0c plugin with the Fiji imagej.net image processing package. This software is able to determine positive and negative nuclei by analysing the image according to brown and blue colour thresholds. During analysis of slides containing large foci of connective tissue or inflammatory cell infiltrate, the image was masked so that it only contained tumour cells for the recognition analysis.

The third method of assessing Ki67 staining proportion was a grid counting (GRID) according to the description by Vörös et al, thus all images were exported to Microsoft PowerPoint, and a grid of horizontal lines 1.5 cm apart, starting from the middle of the slide in both directions, were spread over each image. The nuclei being intersected by these grid lines were then counted manually and the ratio of positive to all cells (i.e. the Ki67 labelling index [LI]) was calculated.

By the end, for each tumour, there were three pairs of CNB and EXC Ki67 LI values to be correlated with time elapsed after the last cycle of NACT using linear regression models. Concordance between the observation methods was evaluated using Bland-Altman plots.

4. RESULTS

4.1. ASSESSMENT OF CHANGES IN HISTOLOGICAL GRADE AND ITS COMPONENTS FOLLOWING NEOADJUVANT CHEMOTHERAPY

In total, 1257 pairs of CNB and EXC breast cancer specimens could be identified, from which 908 were left after all exclusions. The CHIR group receiving primary surgery consisted of 760 pairs of samples, while the PST group receiving primary systemic chemotherapy had 148 pairs. Taxane chemotherapy was used in the majority of cases in the latter group (132/148, 89.2%) and was generally paired with anthracyclines, mostly epirubicin, or platinum-based agents. In HER2 positive tumours (i.e. HER2-overexpressing or *ERBB2*-amplified), anti-HER2 treatment was also generally administered.

When comparing the distributions of the subscores and grade values of the CHIR and PST groups, in the case of CNBs significant differences could be observed for all parameters (T, P, M and G, all $p < 0.001$), whereas in EXC specimens T ($p < 0.05$), P ($p < 0.001$) and G ($p < 0.001$) were significantly different, but M ($p = 0.544$) was not.

Regarding the change of subscores and grade from CNB to EXC, a high concordance rate was observed in both the CHIR (T: 78.9%, P: 68.9%, M: 74.6%, G: 71.2%) and PST (T: 77%, P: 70.3%, M: 50%, G: 61.5%) groups. In the discordant cases, the most common change was that of a difference of one score. The concordance rates were significantly different between the CHIR and PST groups in G ($p = 0.024$) and the M subscore ($p < 0.0001$), but not in the T ($p = 0.68$) and P ($p = 0.82$) subscores. For discordant cases, the following trends could be identified. Changes in the subscore of T were predominantly from a low tubule formation to a higher tubule formation tendency (i.e. from high to a lower subscore) in both groups. In the CHIR group, the most commonly observed changes in P and M were that of a single point increase (1➔2 or 2➔3), however in the case of M, 2-point increases were also present. In the PST group, the P subscore tended to increase (2➔3), however the dominant change in M subscore was a single- or 2-point-reduction. As a result of the changes described above, the grade more commonly increased in the CHIR (71.2%, 95% CI: 64.7%-77.0%) and decreased in the PST (63.2%, 95% CI: 50.2%-74.5%) groups (Fig.1). The results of the Wilcoxon signed-rank test indicate a statistically significant change across all evaluated parameters in both groups (CHIR group: T, P, M and G, all $p < 0.001$; PST group: T, $p < 0.001$, P, M and G, $p < 0.05$).

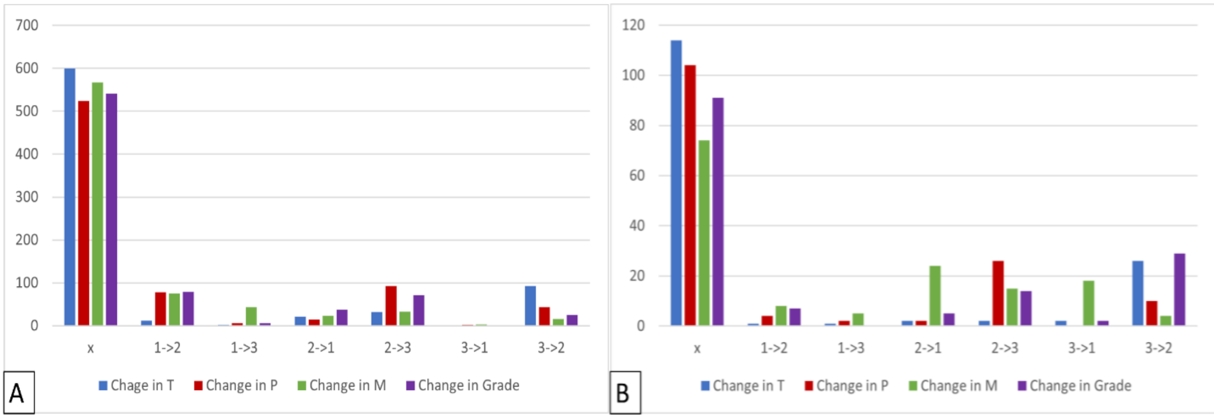

Figure 1. Changes in the values of T, P, M subscores and grade (G) from CNB to EXC in the CHIR (A) and PST (B) patient groups.

CHIR: the group treated with primary surgery; CNB: core needle biopsy; EXC: excision specimens; G: histological grade; M: mitotic activity; P: nuclear pleomorphism; PST: the group treated with primary chemotherapy before surgery; T: tubule (gland) formation; x: no change in the values

4.2. EXAMINATION OF THE PROGNOSTIC VALUE OF POSTNEOADJUVANT GRADE

Altogether 355 patients were included in this study, all of whom were diagnosed with breast cancer, received NACT and residual invasive carcinoma was present in their excision specimens. The most common tumour type was breast carcinoma of no special type (NST), ER-positive, BxG 3. Most common type of treatment was taxane-based NACT and mastectomy. Approximately 40% of patients were diagnosed with ypT1 and 70% of patients with ypN1-3 categories, yG1 was barely identified, while the frequency of yG2 and yG3 categories was similar. The median time of follow-up was 73 months (range: 3.5-236 months), 86 patients died and 155 experienced disease recurrence.

The highest rate of change was observed in HR-positive, HER2-positive tumours (chi-square: 22.26, degrees of freedom: 3, $p < 0.0001$), followed by HR-positive, HER2-negative cases. When all luminal-like tumours were combined, their proportion of grade change remained significantly higher than that of triple-negative or HER2-positive, HR-negative breast cancers (chi-square: 11.32, degrees of freedom: 2, $p = 0.004$). Several of the differences in both OS and RFS across the different comparisons of BxG and yG categories were statistically significant. In general, higher grade categories of both BxG and yG were correlated to unfavourable prognoses regarding RFS and OS estimates, respectively. Subgroup analysis was carried out for cases with different BxG and yG, which indicated an adverse impact of higher yG categories on both RFS and OS estimates (Fig. 2). Out of all cases, both BxG and yG data were available for 320 tumours; there was no change in grade in 221 (69%) cases, while an increase could be observed in 21 (7%) and a decrease in 78 (24%) cases.

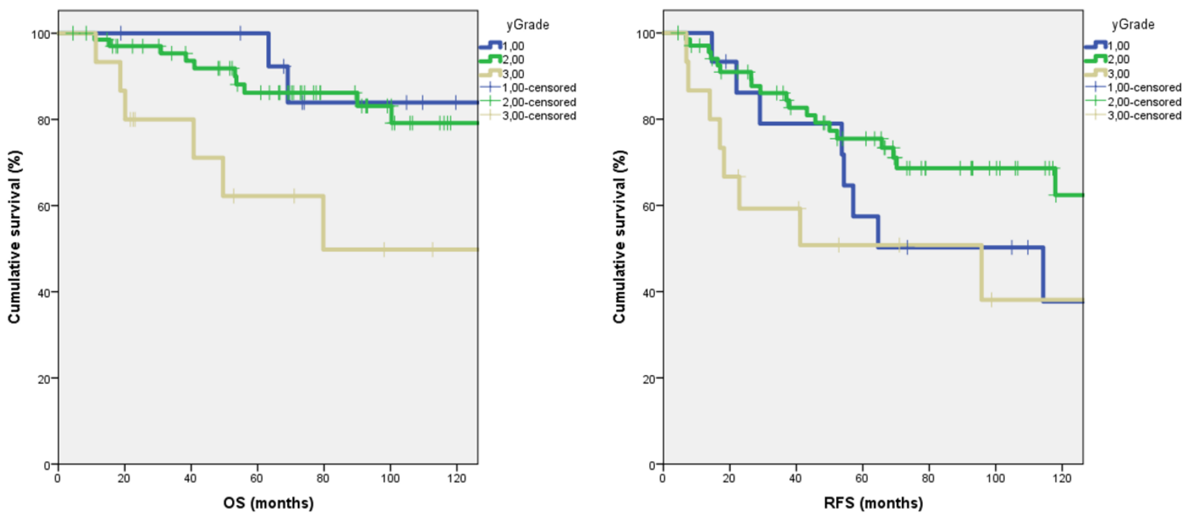

Figure 2. Subgroup analysis of the cases where BxG and yG were different (n=99) and Kaplan-Meier survival curves. Significant differences were found between RFS estimates of yG 2 vs. 3 (p_{RFS}=0.022), but not between yG 1 vs. 2 (p_{RFS}=0.213) and yG 1 vs. 3 (p_{RFS}=0.463). Furthermore, significant differences were found between OS estimates of yG 1 vs. 3 (p_{OS}=0.048), and yG 2 vs. 3 (p_{OS}=0.008) but not between yG 1 vs. 2 (p_{OS}=0.555), respectively.

In univariate analysis older age, lack of ER and PR expression, presence of lymphovascular invasion, incomplete resection and higher ypT, ypN and yG categories were significant adverse prognostic parameters regarding OS, while lack of ER and PR expression, mastectomy, presence of lymphovascular invasion and higher ypT, ypN, BxG and yG categories proved to be significant prognosticators of unfavourable outcome regarding RFS. In multivariate analysis, concerning OS and RFS estimates, significant independent prognosticators were ER status (HR_{OS}: 2.71, 95% CI: 1.74–4.20,

p_{OS}<0.001; HR_{RFS}: 1.72, 95% CI: 1.14–2.59, p_{RFS}=0.009), lymphovascular invasion (HR_{OS}: 2.26, 95% CI: 1.42–3.58, p_{OS}=0.001; HR_{RFS}: 1.84, 95% CI: 1.27–2.69, p_{RFS}<0.001) and the ypN category (HR_{OS}: 3.32, 3.50 and 5.11 with p_{OS} all <0.001 and HR_{RFS}: 2.46, 3.06 and 3.16 with p_{RFS} all <0.001 for ypN1a, ypN2 and ypN3, respectively), respectively. Subgroup analysis was carried out for cases with different BxG and yG categories and univariate analysis indicated older age (HR_{OS}: 5.83, 95% CI: 1.69–20.06, p_{OS}=0.005) and higher yG category (HR_{OS}: 5.13 95% CI: 1.03–25.88 p_{OS}=0.045) had an adverse impact on prognosis. None of them proved to be independent in multivariable analysis. Among HR-positive, HER2-negative breast cancer cases, significant differences were found between the RFS and OS estimates of yG1 vs. yG3 and yG2 vs. yG3 categories (p_{RFS} yG1 vs. yG3 = 0.021; p_{RFS} yG2 vs. yG3 = 0.002; p_{OS} yG1 vs. yG3 = 0.005; p_{OS} yG2 vs. yG3 = 0.001).

4.3. DESCRIPTION OF THE CHANGE IN KI67 LABELLING INDEX FOLLOWING NEOADJUVANT TREATMENT AND ITS TEMPORALITY

Following exclusions, a total of 54 paired cases were included in the study. The most common surrogate molecular subtype observed was luminal B-like (n=22), followed by TN (n=16), HER-positive (n=12) and lastly luminal A-like (n=4). The most common regression grades were RCB-II (n=39) and TR2b (n=20). The average time between the last cycle of NACT and surgical operation was 40.3 days (range: 8-82). Taxane-containing NACT regimens were used in 52/54 cases and it most often involved the sequential administration of epirubicin and docetaxel, however, some patients received a taxane and platinum combination regimen or other taxane-containing regimens. Anti-HER2 treatment in the form of trastuzumab (5 ER+ and 1 ER- tumours) or a dual blockade with trastuzumab and pertuzumab (4 ER- tumours) was administered in 10/12 patients with HER2-positive tumours.

All cases were reported by the same pathologist and the slides reported by others were reviewed by the same pathologist (GCs) to limit interobserver variability. The same microscope was used for scoring mitoses during the period of data collection.

In the linear regression model of Ki67 LI changes over time, before subgroup analysis, the best goodness-of-fit was achieved by the EB-EST method, indicating an upward trend and thus a decreasing change in Ki67 LI over time ($p=0.003$, $R2=0.1548$), therefore, this method was selected for further comparisons; the widespread use of this method was also considered (Fig. 3).

In the subgroup analysis based on surrogate molecular subtypes, HER2-positive ($p=0.091$, $R2=0.2589$), TN ($p=0.045$, $R2=0.2563$) and Luminal B-like ($p=0.039$, $R2=0.1961$) tumours all demonstrated an upward trendline, suggesting that the proliferation blocking effect of NACT in these tumours decreases over time. Luminal A-like tumours exhibited the best fit with the linear regression model ($p=0.125$, $R2=0.766$), indicating a decreasing trendline and thus an increase in the proliferation blocking effect as time passes following last cycle of NACT, albeit this subgroup contained the fewest number of cases ($n=4$), and therefore no conclusions can be drawn for this subgroup.

Subgroup analysis based on RCB regression grade scores demonstrated discordant trendlines between RCB-I versus RCB-II and RCB-III categories, with the former showing a downward trend and the latter two indicating the inverse; the upward trend, meaning a decrease in proliferation reduction following NACT. The subgroups RCB-I and RCB-III had a suboptimal number of cases ($n=8$ and $n=7$, respectively), a known limitation of our study.

Lastly, in the subgroup analysis based on the EWGBSP TR regression categories the linear regression trendline for TR2a tumours showed a downward trend and thus a sustained effect of chemotherapy ($p=0.276$, $R2=0.2819$), while TR2b ($p=0.051$, $R2=0.1951$), TR2c ($p=0.028$, $R2=0.3209$) and TR3 ($p=0.02$, $R2=0.4019$) groups all demonstrated an opposite, upward trend.

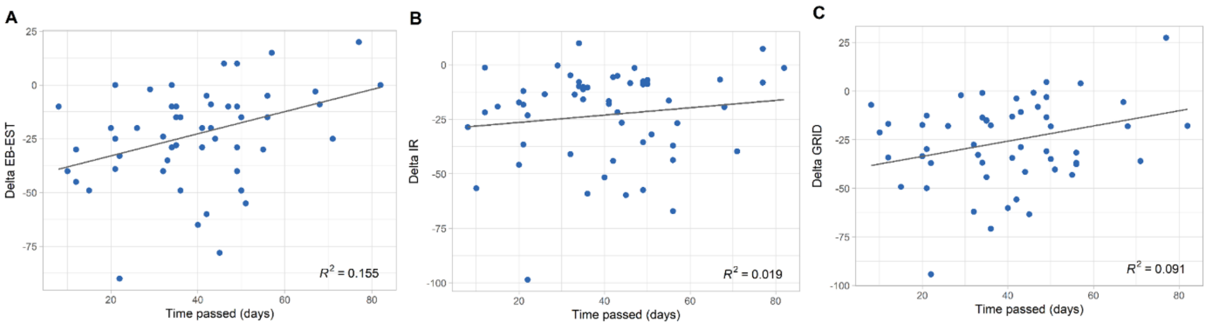

Figure 3. Linear regression results and R^2 values for all cases, according to quantification method. Delta: difference between post- and pretreatment Ki67 LI values; EB-EST: eyeballing-based estimation method, IR: ImmunoRatio software results; GRID: gridline-based counting and proportion of positive cells

In core biopsies, the Bland-Altman analysis assessing the agreement between the EB-EST and IR, and the EB-EST and GRID (in this order, pairwise) estimation methods showed a mean difference (bias) of 9.3 (95% CI: 4.6 to 14.0; p<0.001) and -4.0 (95% CI: -7.3 to -0.6; p=0.02), with limits of agreement ranging from -24.6 to +43.2 and -28.1 to +20.2, respectively.

As for the values in excision specimens, the Bland-Altman analysis demonstrated an average difference of 9.8 (95% CI: 5.1 to 14.6; p<0.001) and -0.9 (95% CI: -3.0 to +1.2; p=0.4) between EB-EST and IR, and EB-EST and GRID, respectively. The ranges for limits of agreement were -24.2 to +43.9 and -15.9 to +14.1, respectively. Additionally, significant increasing trends in the differences were detected across the range of methods (p<0.001 and p=0.01, respectively), which were not present in the core biopsy results (p=0.874 and p=0.112, respectively). Specifically, the difference between the estimation methods increases with the magnitude of the Ki67 LI value – that is, EB-EST tends to produce higher values than IR or GRID as values increase.

5. DISCUSSION

The traditional prognostic factors of breast cancer, including histological grade, remain relevant even in the era of molecular and genomic testing, and histological grade is considered a good prognosticator despite interobserver variability. A higher BxG (i.e. G3) is associated with pCR following NACT, which is an independent prognosticator based on multivariate analysis. Previous studies demonstrated that in spite of concordance in grade in preoperative and operative samples in most of the cases, discordant cases show some trends. A meta-analysis based on 33 studies (4980 patients) suggested concordance in grade in the majority of cases (range: 59% to 94%, pooled estimate: 71%), however, in discordant CNB and EXC grades, it indicated an underestimation twice as commonly (19%) as overestimation (9%). Some of the included studies also reported on the subscores for grade calculation, and in discordant cases, specific trends could be observed in these as well: 1./ Concordance was predominant, but when faced with discordance, 2./ Tubule formation (T) was more commonly overscored than underscored (13% vs 9% based on pooled percentages of 12 studies), 3./ Nuclear pleomorphism (P) was more likely to be underestimated than overestimated (17% vs 10% based on 14 studies), 4./ Lastly, mitotic activity (M) was more often underestimated than overestimated (30% vs 8% on the basis of 13 studies). Our results based on the analysis of change in grade and its subscores are in keeping with the pooled analyses presented. Concordance rate for grade between CNB and EXC specimens was 71%, not only nearly identical to the pooled results, but also a reliable data, as the study with the greatest weight included in the meta-analysis included 300 cases, less than half of the number of cases included in the CHIR group of our study. Our data also reinforce the previously described tendency to overestimate T and underestimate P, M and G, with G being nearly twice as often underestimated than overestimated.

Discrepancies between grades determined from CNB or EXC specimens have generally been explained by underrepresentation by CNB. This effect is even more pronounced in breast cancers that show heterogeneity within the tumour mass, such as mixed tubular carcinomas. Another recognised phenomenon is the zonation of proliferation and the stemming recommendation to count mitotic figures at the periphery of the tumour, as these areas present a more optimal environment for proliferation (i.e. lack of hypoxia).

Both heterogenous tubule formation and zonal distribution of proliferative cells can lead to discrepancies on the basis of tangential versus radial sampling by the needle and these variabilities play a lesser role in EXC specimens where we can better observe the complete cut surface of the lesion. As the size and quality of the CNB sample (at least 2

mm^2 tumour area, 10 high power fields, no crush artefacts) are also important to be able to adequately determine grade, these samples may end up being unassessable. Differences in cold ischaemia time and fixation are also mentioned as a possible cause for discrepancies. Less than perfect interobserver variation has also been mentioned as a possible cause of discrepancies, since the reproducibility of histological grading of breast cancers has been found to be only moderate, however, interobserver variability did not influence the results of our study, as all cases have been reported by the same pathologist, and intraobserver agreement of grading has always been reported as better than interobserver agreement. All aforementioned phenomena should play a role in discrepancies seen in the PST group and dissonant trends may be attributed to the differences of the populations or as effects of NACT. In the PST group, while the majority of cases still demonstrated concordance, the rate was significantly lower than in the CHIR group (71% vs 62%). In discordant cases, the most notable change was that of a decrease in G (88%). Regarding the subscores, T and P indicated a substantial concordance between CNB and EXC samples and the rates were nearly identical to those observed in the CHIR group. By contrast, the M subscore demonstrated discordance in half of the cases, significantly more often than in the CHIR group. For discordant cases, T and M scores more often decreased, whereas P more commonly increased. Taxanes may cause the formation of pleomorphic tumour giant cells and bizarre, large nuclei by disrupting microtubule function and mitotic spindle formation, ultimately increasing pleomorphism subscore, however, this might not play a significant role in the changes observed, as most of the cases were originally of P score 3. Changes in the M subscore may be attributed as the desired and expected effect of NACT. As a combined result of the above-mentioned effects and especially of the drastic decrease in M subscores in the PST group, G tended to decrease rather than increase in the EXC specimens, an effect attributable to NACT.

As most breast cancers treated with NACT are of high grade, and reflect subscores 3 for most assessed parameters, aggravation cannot occur in most cases, but a reduction in proliferation can lead to decreased grade in the residual tumour. Whether this change is associated with improved prognosis is not well known, nonetheless, reporting of histological grade following NACT is part of several recommendations. The extent of residual tumour following NACT has been proven to be of prognostic relevance as reflected by RCB, but the addition of Ki67 proliferation index, ER status and yG further improves prognostication, suggesting that yG has prognostic importance, too. It should be noted, that the cases analysed in our study have not reached pCR, and therefore represent a cohort with worse prognosis. In our study comparing grades of CNB and EXC samples, we noted a decrease in grade in 24% of tumours following NACT, and in our second study, grade decreased comparably in 24%. A previous study based on the results of 485 patients treated with anthracycline or anthracycline + taxane based NACT indicated the prognostic impact of both BxG and yG, with the impact being greater in the yG setting. The cohort included 8% G1, 41.5% G2 and 50.5% G3 tumours, pCR was achieved in 115 cases (23.7%). The remaining 370 cases with residual disease showed a split of 12% yG1, 55% yG2 and 33% yG3 and the Cox proportional hazard model showed a significant overall and distant disease-free survival disadvantage for yG3 (but not yG1 and yG2) compared to pCR, though yG was not assessed separately for cases with residual tumours only.

As our previous results demonstrate, the leading factor in grade reduction following NACT is the marked reduction in the M subscore, that is, proliferation of the tumour. The reaction of the tumour to NACT can be described as heterogeneous, as its highly dependent on the biological behaviour of breast cancer, supported by the fact that it is more often given to patients with poorly differentiated, highly proliferative tumours, as these patients may benefit more from the proliferation blocking effects of NACT.

The importance of Ki67 may arise during response-driven therapy adjustment regimens, as well as subdivision of breast cancers into surrogate molecular subtypes.

Our results based on mitotic counts indicate and underestimation of proliferation in discordant cases following surgery without preoperative systemic therapy, while in NACT cases, proliferation tended to be higher in core biopsies than in surgical specimens, a result that is in keeping with the literature.

While ΔKi67 is also recognised as prognostic, yKi67 remains the main factor predicting early local and distant metastasis risk and survival, with higher yKi67 values corresponding to poorer prognosis, regardless of the extent of the change. Many authors seem to agree on the prognostic value of yKi67 LI.

We may conclude from previous studies that Ki67 LI and its change carry important information and has an impact on prognosis, and yet, the temporality and trend of its change over time between the last cycle of chemotherapy and surgery remain to be less investigated. Our study aimed to fill this gap in knowledge. Our cumulative data may suggest that the drop in proliferation after NACT may gradually be lost, and therefore, time from the last chemotherapy administration may also influence the prognostic value of yKi67 and ΔKi67. This suggestion would be more straightforward if all tumours behaved similarly to NACT, which is not the case. When we tried to analyse subsets of tumours according to their surrogate molecular classification or the degree of response to NACT, we saw a similar tendency for a drop in yKi67 in nearly all subsets, except for the good prognosis luminal A-like tumours and the tumours with the best response to treatment (RCB class I and EWGBSP TR2a), all underrepresented in the cohort. The data are purely descriptive and seem to reflect a dynamism in the proliferation-reducing effect of NACT. We used different methods of assessment, including two objective ones based on sampling (IR, GRID) and a thoroughly carried out but subjective one (EB-EST), and all showed similar trends; we have finally selected EB-EST based on the best goodness-of-fit and its widespread use in clinical practice.

6. CONCLUSIONS AND NEW FINDINGS

Our results reinforced the previously described concordance of grade and its subscores between CNB and corresponding EXC specimens, as well as the tendency to underestimate tubule formation (by overestimating its score), nuclear pleomorphism, mitotic rate and grade on CNBs when compared with EXC samples in discordant cases. Following primary systemic chemotherapy, concordance rates were lower and a reduction in M subscore and grade could be observed.

Our second work strengthened the prognostic value of grade in the postneoadjuvant setting, indicating that yG3 is associated with worse RFS and OS than yG1 and yG2, therefore a reduction in grade seen in approximately 1/4 of the cases as demonstrated by both our first and second study may be associated with better prognosis. This result was supported by the univariable Cox regression analyses, however, as the significance is lost in multivariable analysis, the prognostication does not seem to be independent.

Our findings reinforce a drop in proliferation following NACT in most breast carcinomas evaluated, and suggest that this drop may decrease with increasing time between the last cycle of NACT and surgery. This dynamism was seen in more aggressive molecular subtypes and tumours with lesser response to treatment, and might be missing from the rare luminal A-like tumours or carcinomas with better response to treatment, though conclusions for these latter subgroups were limited by low case numbers.

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