APPENDIX

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

IF (2024): 2,9 (Scimagojr: Q1)

PATHOLOGICA 2024;116:22-31;
DOI: 10.32074/1591-951X-958

Original Article

Changes in breast cancer grade from biopsy to excision following surgery or primary chemotherapy

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Summary

Objective. To compare histological grade (G) of breast cancer and its components (scores for tubule formation - T, nuclear pleomorphism - P and mitotic counts - M) in core needle biopsies (CNBs) and surgical excision specimens (EXC) in patients treated with primary surgery (CHIR) or primary chemotherapy (PST).

Methods. Grade of matched pairs of carcinomas in CNB and EXC was assessed according to the Nottingham grading system.

Results. PST cases tended to have higher pretreatment G. Concordance rates in the CHIR (n = 760) and PST (n = 148) groups for T, P, M and G were 79%, 70%, 75%, 71% and 77%, 70%, 50%, 62%, respectively; differences in concordance rates were significant in M ($p < 0.0001$) and G ($p = 0.024$). For discordant cases in the CHIR group, CNBs tended to overestimate T and underestimate P, M and G, whereas in the PST group, the same trends were identified for T and P, but there was a significant tendency for M and G to be lower in EXC specimens.

Conclusions. The reversal of M and G underestimation in CNB to "overestimation" in the PST group can only be explained with the effect of mitosis reduction following chemotherapy. Whether the posttreatment decrease in G reflects any prognostic value remains to be elucidated.

Key words: Breast cancer, histological grade, core needle biopsy, excision, neoadjuvant chemotherapy

Received: December 8, 2023
Accepted: December 11, 2023

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How to cite this article: Ferenczi A, Cserni G. Changes in breast cancer grade from biopsy to excision following surgery or primary chemotherapyr. Pathologica 2024;116:22-31. https://doi.org/10.32074/1591-951X-958

Introduction

Histological grade of invasive breast carcinomas, as modified by Elston and Ellis, i.e., the so-called Nottingham grade is a prognostic factor of proven value [1], and has become a standard part of histology reporting of breast cancer, including the International Collaboration on Cancer Reporting (ICCR) dataset recommendations [2]. As all pathologists know, this is a combined grading system that incorporates lumen (tubule, gland) formation, nuclear pleomorphism and area adjusted mitotic rate, all of which are scored on a three-tiered ordinal scale, and give a sum that is used to determine the grade, which is itself an ordinal variable in prognostication. The prognostic impact was first established on the basis of patients treated by primary surgery, therefore surgical specimens were used for grading, and form the gold standard [1].

A criticism of histological grading is that all of its elements are subjectively evaluated, and are influenced by the grader's experience and skills. The interobserver consistency, reproducibility of this parameter is generally considered moderate [3,4]. Nevertheless, its prognostic impact

is unquestionable even in the era of gene expression profiling, as reflected by its added value to genomic markers of prognosis [5,6].

Grade of breast cancers is also assessed in core needle biopsies (CNBs). In some cancers, this is the only tumour sample allowing the assessment of prognostic and predictive markers, as patients do not have residual tumour following primary systemic treatment, or there is no further specimen to assess after some novel/experimental forms of local treatment such as cryoablation [7], radiofrequency ablation [8] or high-intensity ultrasound ablation [9]. As one or a few tissue cores are only a limited representation of the tumour, their representative value has been tested in some studies and has been found to increase with the number of tissue cores obtained [10]. Overall, histological grade, as assessed on CNB, seems a moderate reflection of tumour differentiation established on the basis of excision specimens with 71% (95% confidence interval (CI): 69-73%) pooled agreement between the two, and a pooled estimate of Cohen's kappa of 0.54 (95%CI: 0.50-0.58) [11].

The ICCR also recommends to determine histological grade in the post-neoadjuvant setting, although the impact of grade on prognosis is based on less evidence in this context [12].

In the present study, we aimed to compare the grade and its components as determined in paired CNB and surgical excision (EXC) specimens in patients who were treated by primary surgery (CHIR) and those who received neoadjuvant chemotherapy (PST) and had residual cancer to grade.

Materials and methods

Invasive breast cancers diagnosed at the Bács-Kiskun County Teaching Hospital between 2010 and 2022 with available preoperative CNB and EXC specimens were selected for this study. All CNB samples were gained by image-guided (generally ultrasound-guided) 14G gun biopsies with the aim of obtaining 3 tissue cores. Of the cases with this dual specimen representation at the Department of Pathology, the following were excluded: i) multifocal tumours where the histological grade of the foci was different, and it could not be decided which focus was sampled preoperatively (cases with identical histological grade of the multiple foci sampled were not excluded); ii) the CNB specimen was crushed or had limited diagnostic value, preventing adequate assessment of grade – e.g., tumour dimension smaller than 10 high power fields (2 mm^2) or uncertain nuclear pleomorphism or lumen formation due to crush artefacts; iii) the CNB diagnosis was that of an in situ carcinoma; iv) neoadjuvant endocrine therapy was given; v) pathological complete regression was achieved or the residual disease was not suitable for grading.

In all included cases, the histological grade and subscores for its components were extracted from the histology reports. All data were recorded in Microsoft Excel spreadsheets.

Histological grade was assessed according to the Nottingham scheme also recommended by the most recent World Health Organization (WHO) Classification of breast tumours by one of two pathologists with experience in breast pathology [13]. For each pair of samples, the three-tiered values of the histological grade (G) and its subscores for tubule/gland formation (T), nuclear pleomorphism (P) and standardised mitotic rate (M) were recorded for the CNB specimen first, and these were coupled with the corresponding values from the EXC specimen as a second measurement of the same parameters.

The distributions of each value for the T, P and M subscores and histological grade in the CHIR and PST groups as well as the concordance rates in the two groups were compared by means of the Chi-square test for both CNB and EXC specimens.

To assess the changes in values of T, P and M subscores and G from CNB to EXC in the CHIR and the PST groups, the nonparametric Wilcoxon signed rank test was used as this accounts for positive and negative changes of the variables, and helps to check whether the changes seen are random or show any tendency. The calculations were done in the Microsoft Excel's Real Statistics Resource Pack add-in, with the corrections recommended by the creator and author of the add-in [14,15]. For the statistical tests, a significance level of $p < 0.05$ was used.

The study was approved by the Human Investigation Review Board, University of Szeged (approval number 90/2021-SZTE-RKEB).

Results

A total of 1257 pairs of invasive breast cancer CNBs and EXC specimens were identified, from which 908 were left after the exclusions. Of these, 760 did not receive neoadjuvant chemotherapy (CHIR group), whereas 148 patients received such a preoperative treatment (PST group). The primary systemic therapy in the latter group often included a taxane which was part of the treatment in 132/148 (89.2%) cases, and was generally administered consecutively with anthracyclines (most commonly epirubicin) or a platinum derivative. Anti-HER2 treatment was also included for

HER2 positive (overexpressing or amplified) tumours. The distribution of grading subscores and histological grades in the various groups are shown in Table I, and Figure 1.

Comparing the distributions of the grading subscores and the grades of the CHIR and PST groups, there were significant differences in all parameters in CNB specimens (T, P, M and G, all $p < 0.001$), whereas only T ($p < 0.05$), P ($p < 0.001$) and Grade ($p < 0.001$) showed significant differences in EXCs; the M subscore was not statistically different ($p = 0.544$).

The PST/CHIR ratio of relative frequencies observed in each subscore and grade is represented in Figure 2. I is remarkable for the CNB specimens, that at the time of planning therapy, the PST group shows greater proportions of poorly differentiated (G3) carcinomas, and higher rather than lower mitotic count based-scores. These excesses are no longer seen in the EXC specimens, whereas the higher relative prevalence of P score 3 in the PST group did not change from CNB to EXC.

The changes in grading subscores (T, P and M) and G from CNB to EXC for the CHIR and PST groups are shown in Figure 3. It is apparent that the subscores and grades of the two types of specimens were concordant in the majority of the cases in the CHIR group (T: 78.9%, P: 68.9%, M: 74.6%, G: 71.2%) and the PST group (T: 77%, P: 70.3%, M: 50%, G: 61.5%), and whenever there was discordance, the changes were most commonly of one score. The unchanged parameters are detailed in Table II. Despite the majority of the cases being concordant for the variables assessed in CNB and EXC in both groups, there were significant differences between the concordance rates of the CHIR and the PST samples in G ($p = 0.024$) and its M subscores ($p < 0.0001$), whereas there was no such difference in the T ($p = 0.68$) and P subscores ($p = 0.82$).

For discordant cases, the following trends could be identified. Gland formation (T subscore) changes were predominated by a change from a low CNB tubule formation, i.e. high score (< 10%; T: score 3) to a higher tubule formation tendency, i.e. a lower subscore (10-75%; T: score 2) EXC value in both the CHIR and the PST groups. In the CHIR group, the most common change in both nuclear pleomorphism (P) and mitotic rate (M) was a single point increase (1→2 or 2→3), however in case of M, 2-point-increases were also noted. In the PST group, one of the most common changes was the P: 2→3 increase in pleomorphism, however a single point or a 2-point-reduction of M was the dominant change observed. As a result of these changes in subscores, the histological 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.

The Wilcoxon signed rank test indicated that there was a statistically significant change (from CNB to EXC) in all the parameters evaluated and in both groups (CHIR group: T, P, M and G, all $p < 0.001$; PST group: T, $p < 0.001$; P, M and G, $p < 0.05$).

Table I. Case numbers, (percentages and [95% confidence interval limits]) of tubule formation, nuclear pleomorphism and mitotic activity subscores and histological grades in the two types of specimens in the two groups investigated.

CHIR (n = 760)				PST (n = 148)			
CNB	1	2	3	CNB	1	2	3
T	60 (7.9% [6.2-10.0])	187 (24.6% [21.7-27.8])	513 (67.5% [64.1-70.7])	T	2 (1.4% [0.4-4.8])	20 (13.5% [8.9-20.0])	126 (85.1% [78.5-90.0])
P	127 (16.7% [14.2-19.5])	372 (49.0% [45.4-52.5])	261 (34.3% [32.0-37.8])	P	8 (5.4% [2.8-10.3])	45 (30.4% [23.6-38.2])	95 (64.2% [56.2-71.5])
M	553 (72.8% [69.5-75.8])	94 (12.4% [10.2-14.9])	113 (14.9% [12.5-17.6])	M	59 (39.9% [32.3-47.9])	43 (29.1% [22.3-36.8])	46 (31.1% [24.2-38.9])
G	233 (30.7% [27.5-34.0])	376 (49.5% [45.9-53.0])	151 (19.9% [17.2-22.9])	G	14 (9.5% [5.7-15.3])	63 (42.6% [34.9-50.6])	71 (48.0% [40.1-56.0])
EXC				EXC			
T	68 (9.0% [7.1- 11.2])	238 (31.3% [28.1-34.7]	454 (59.7% [56.2-63.2])	T	4 (2.7% [1.1- 6.7])	43 (29.1% [22.3-36.8])	101 (68.2% [60.4-75.2])
P	60 (7.9% [6.2- 10.0])	386 (50.8% [47.2-54.3])	314 (41.3% [37.9-44.9])	P	4 (2.7% [1.1- 6.7])	31 (21.0% [15.2-28.2])	113 (76.4% [68.9-82.5])
M	461 (60.7% [57.1-64.1])	129 (17.0% [14.5-20.0])	170 (22.4% [19.6-25.5])	M	88 (59.5% [51.4-67.0])	16 (10.8% [6.8-16.8])	44 (29.7% [23.0- 37.5])
G	186 (24.5% [21.6-27.7])	371 (48.8% [45.3-52.4])	203 (26.7% [23.7-30.0])	G	13 (8.8% [5.2-14.5])	81 (54.7% [46.7, 62.5])	54 (36.5% [29.2- 44.5])

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.

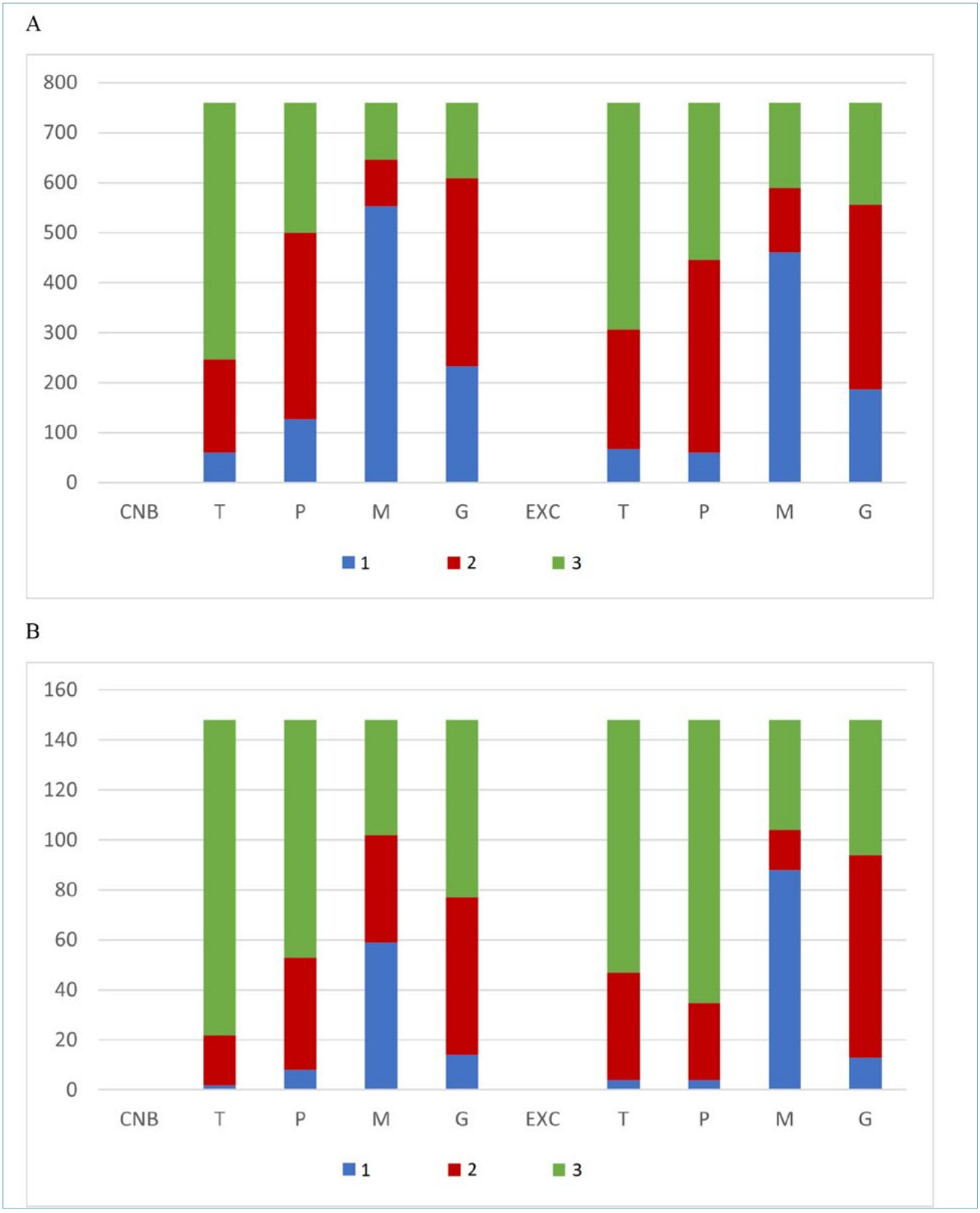

Figure 1. Distributions of case numbers for each grading subscore (T, P and M) and grade (G) in the group treated with A: primary surgery (CHIR) or B: primary chemotherapy (PST). CNB: core needle biopsy, EXC: excision specimen. *CHIR: the group treated with primary surgery, CNB: core needle biopsy, EXC: excision specimens, G: histological grade, M: mitotic activity, P: nuclear pleomorphism, PST: the group treated with primary chemotherapy before surgery, T: tubule (gland) formation.*

Table II. Unchanged parameters from core needle biopsies to excision in the two groups investigated.

CNB to EXC:	1-1	2-2	3-3	All
T (CHIR)	46 (7.7%)	134 (22.3%)	420 (70%)	600
P (CHIR)	43 (8.2%)	265 (50.6%)	216 (41.2%)	524
M (CHIR)	435 (76.7%)	38 (6.7%)	94 (16.6%)	567
G (CHIR)	148 (27.4%)	267 (49.4%)	126 (23.3%)	541
T (PST)	0 (0%)	16 (14%)	98 (86%)	114
P (PST)	2 (1.9%)	17 (16.3%)	85 (81.7%)	104
M (PST)	46 (62.2%)	4 (5.4%)	24 (32.4%)	74
G (PST)	7 (7.7%)	45 (49.5%)	40 (44%)	91

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.

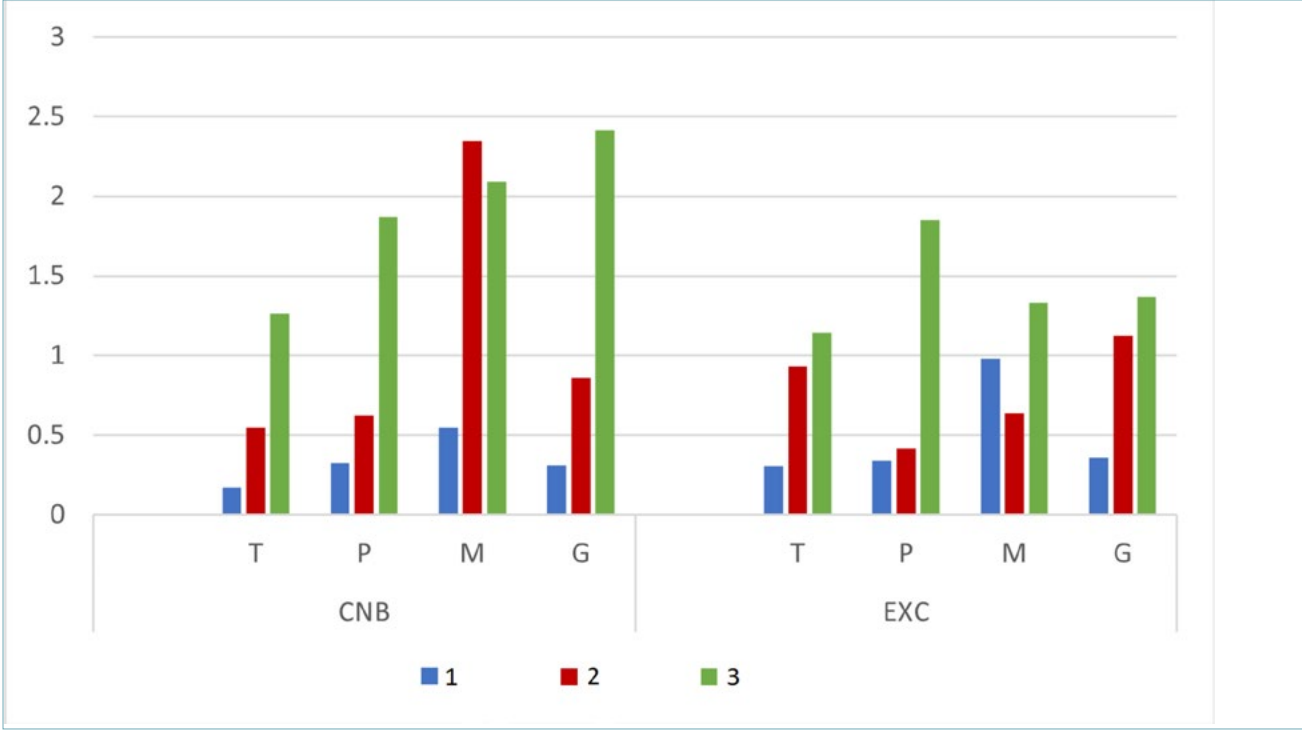

Figure 2. PST/CHIR ratio of relative frequencies per grading subscores (T, P and M) and grade (G). *Values above 1 (especially > 1.5) reflect greater incidence of a variable in the PST group. CHIR: the group treated with primary surgery, CNB: core needle biopsy, EXC: excision specimens, G: histological grade, M: mitotic activity, P: nuclear pleomorphism, PST: the group treated with primary chemotherapy before surgery, T: tubule (gland) formation.*

Discussion

The clinical value of some traditional prognostic factors of breast cancers, like histological type [16] or grade has been scaled back by the possibility to classify tumours according to biomarkers for targetable genetic or phenotypic alterations. Nevertheless, histological grade of breast cancer is a recognised prognosticator of this disease maintaining its utility even in the era of genomic tests of prognosis and despite reported interobserver variability. There have been studies comparing grading on CNB and EXC specimens, and it has been shown that despite being concordant in many cases, there are some trends in discordance. A meta-analysis of 33 studies (4980 patients) suggested that concordance in grade was seen in the major-

ity of cases (ranging from 59% to 94%, with a pooled estimate of 71%), but when the CNB grade was discordant from the EXC grade, underestimation was roughly twice as common (19%) than overestimation (9%) [11]. Some of the studies included in the cited meta-analysis also reported on grade components, and could be assessed for concordance and discordance: 1. concordance was predominant for all parameters, but when discordant, 2. tubule formation (T) was more frequently overscored than underscored (13% vs 9% on the basis of pooled percentages of 12 studies); 3. nuclear pleomorphism (P) was more often underestimated than overestimated (17% vs 10% on the basis of 14 studies); 4. finally, mitotic activity (M) was more commonly underestimated than overestimated (30% vs 8% on the basis of 13 studies) [11].

Our findings are in keeping with the pooled analysis in many respects. The concordance rate of grade between CNB and EXC specimens was 71%, practically identical with the pooled results, and this is a reliable record as even the study given the greatest weight [17] in the meta-analysis of Knuttel et al. [11] had only 300 cases, i.e. less than half of the cases included in the CHIR group of the present study. The tendencies to overestimate T and underestimate P, M and G are also consistently reinforced by our data; G being roughly twice as often underestimated than overestimated. Concerning the subscores on grade components, we also noted the highest concordance rate for T (79% vs 78%); but there was a minor discrepancy from the meta-analysis data; our second highest rate of concordance was seen in connection with M (75% vs 62%) and this was followed by P (69% vs 73%).

Discrepancies between grades established on CNB and corresponding EXC samples have generally been explained with undersampling by CNB and the ensuing underrepresentation. Indeed, some breast cancers show heterogeneous aspects in appearance. A typical example of this phenomenon may be illustrated by tubular mixed carcinomas, of which the one part, generally the central area, reflects a tubular carcinoma with plenty of tubules, whereas the periphery might not show tubule formation at all (Fig. 3A-C) [18]. Another recognised phenomenon present in many breast carcinomas is the zonation of proliferation, and the stemming recommendation to preferably count mitotic figures at the periphery of the tumours (Fig. 3D). Both of these examples can lead to discrepancies on the basis of radial versus tangential sampling by the needle, but obviously neither sampling passes of the needle will adequately reflect a full cut surface of an EXC specimen. The smaller size or crush artifacts of CNB samples may also lead to the unassessability of grade; e.g., due to the lack of 2 mm^2 tumour area (10 high power fields) for the proper determination of the mitotic score. These cases have been excluded from the present study, too (as grade X cases), though in real life conditions, these are given a likely grade or an approximation such as non-high grade on the basis of the available subscores, to allow the consideration of the limited prognostic information gained from the specimen. Differences in cold ischaemia time and fixation are also mentioned as a possible cause for the discrepancies. Although the EXC samples are considered the gold standard, CNBs may have more ideal tissue processing. Lehr and colleagues have rather confidently explained why larger specimen size, slower permeation by formalin, delayed fixation and the development of more easily identifiable mitotic figures may be the principal cause for the consistent underestimation of M in CNB samples, or more precisely an overestimation in EXC specimens [19]. It is unknown whether alternative fixatives, like acid-free glyoxal [20,21] would have the same effect on M scores, and this remains to be explored.

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 [3,4]. Reflecting our results on concordance of the grade components between CNB and EXC samples, T has been the most consistently and P the least consistently reported component of grade in a large review of the UK breast pathology external quality assessment scheme [22]. However, interobserver variability might have had little influence in the present dataset, as only 6.7% of the CNB/EXC pairs had been independently reported by two different pathologists. Intraobserver variability might have contributed to the discrepancy rates, but intraobserver agreement of grading has always been reported as better than interobserver agreement [23].

All the phenomena described above should play a similar role when assessing the grade of CNB specimens in the PST group, and divergences in the CNB-EXC pairs from trends seen in the CHIR group should either stem from differences in the two populations or the effect of systemic chemotherapy.

Although concordance between CNB- and EXC-assessed histological grade was also seen in the majority of the PST cases, this was significantly less common than in the CHIR group (71% vs 62%). In cases of discordance, the most notable change was that of a decrease in G (88%). Regarding the subscores of grade, T and P presented a substantial concordance between CNB and EXC samples, and both were nearly identical with the corresponding concordance rates observed in the CHIR group (T(PST): 77% vs T(CHIR):

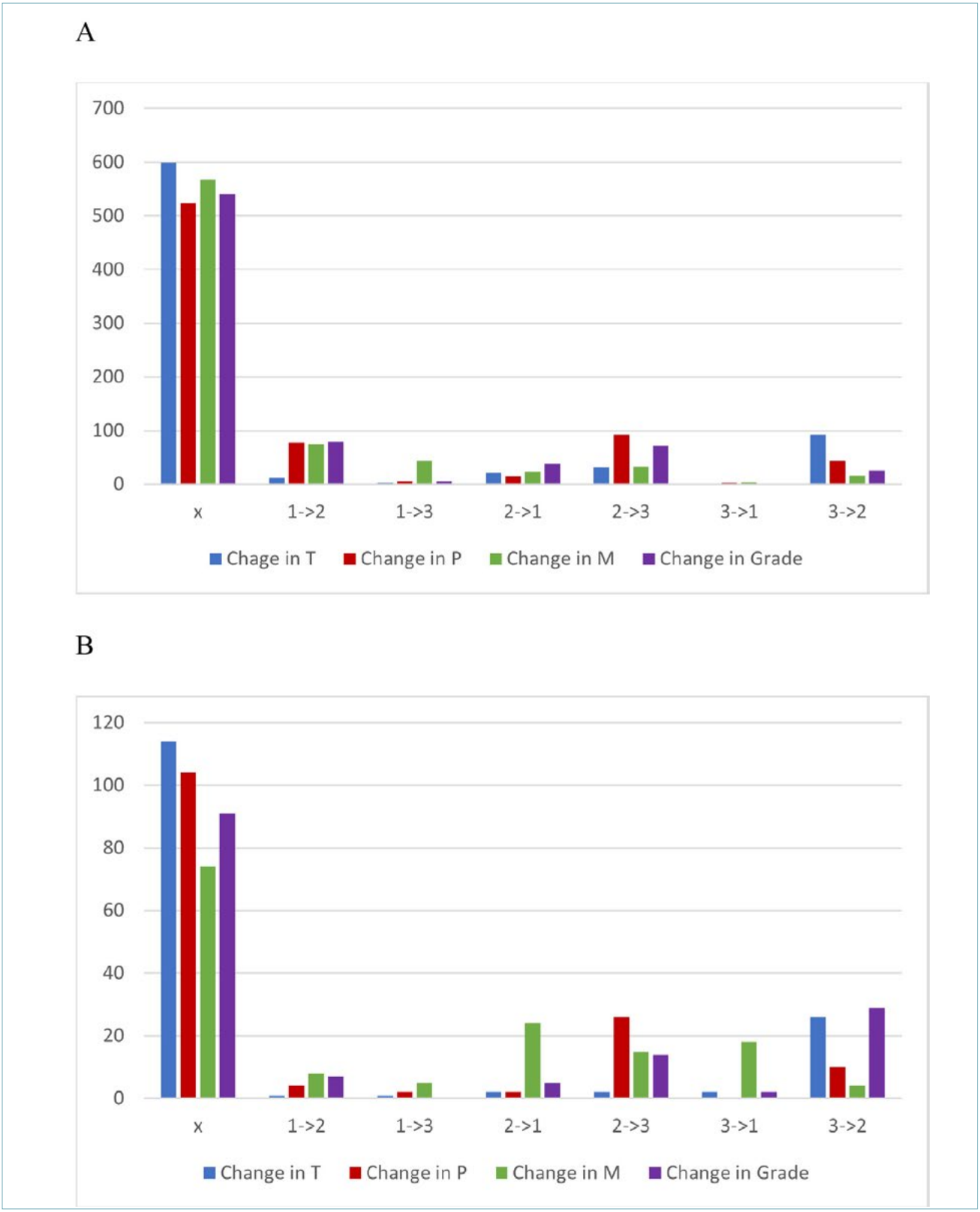

Figure 3. Changes in the values of T, P, M subscores and grade (G) from CNB to EXC in the two patient groups (A: CHIR and B: PST groups).
CHIR: the group treated with primary surgery, CNB: core needle biopsy, EXC: excision specimens, G: histological grade, M: mitotic activity, P: nuclear pleomorphism, PST: the group treated with primary chemotherapy before surgery, T: tubule (gland) formation, x: no change in the values.

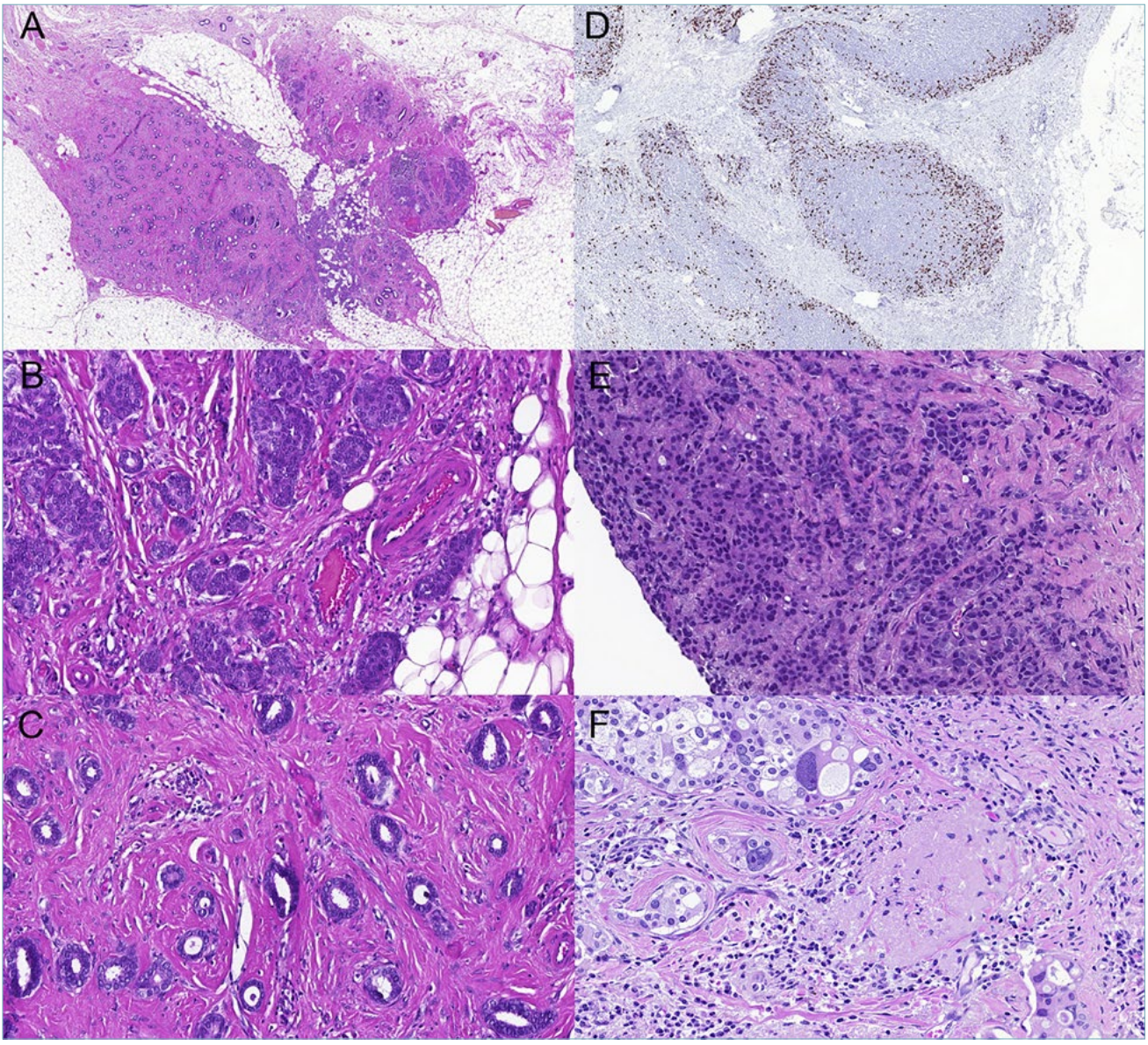

Figure 4. Histological variability that may account for differences in subscores and grades between core needle biopsies and excisions. (A-C): Mixed tubular carcinoma; A: x2, right side with less than 10% gland formation (B: x20) and left side with 100% tubule formation characteristic of tubular carcinoma (C: x20). D: A Ki-67 immunostain highlighting inhomogeneity and zonal distribution of the cells in the cell cycle, a zonation that may also characterise the distribution of mitotic figures (Ki67, x5). (E-F): The same tumour before chemotherapy in the core needle biopsy and after chemotherapy in the excision, with P scores 2 and 3, respectively (x30).

79% and P(PST): 70% vs P(PST): 69%). In contrast, the M subscores were concordant/discordant in half of the cases, and this meant a statistically significant difference from the 75% concordance rate in the CHIR group. For discordant cases, T and M scores more often decreased (in 88% and 62%, respectively), whereas an increase was seen more commonly for P (73%).

The fact that T and P scores showed nearly identical concordance rates in CHIR and PST cases, and discordant cases in the PST group showed overestimation of T and underestimation of P in CNBs similar to that seen in the CHIR cases, suggests the same underlying mechanisms, i.e., undersampling of heterogenous tumours. There is no data on neoadjuvant

treatment affecting gland formation in breast cancer, but there is observational evidence that taxane-containing chemotherapy used in most PST patients here, may lead to the formation of pleomorphic tumour giant cells (Fig. 3E-F) [24]. Taxanes distrupt microtubule function and mitotic spindle formation, leading to polyploidisation and the development of large nuclei with bizarre morphology, which increase pleomorphism [24,25]. However, as many of the tumours were originally of P score 3, such nuclear alterations, might have led to less effect in this population. Changes in the M scores were the most obvious, and these must be related to the mitotic activity blocking effect of the chemotherapy regimen, a desired effect of (neoadjuvant) systemic treatment.

As a combined result of the above-mentioned effects, and especially of the predominant decrease (rather than the increase seen in the CHIR group) of M scores in non-concordant CNB-EXC pairs, G tended to decrease rather than increase in EXC specimens, an effect that we must attribute to the effect of systemic treatment.

A previous study on 40 matched pairs of pretreatment and posttreatment grades also found a significant decrease in the mitotic count score, but not in grade [26].

Little is known about the prognostic role of postneoadjuvant grade, although its determination is a core element of the ICCR recommendations for breast cancer reporting after primary systemic treatment. Physicians from the MD Andreson Cancer Center have reported that nuclear grade 3 has not only a value in predicting the response to neoadjuvant treatment, but also has an additive prognostic value for determining 5-year outcomes; however, the grade used was derived from the preoperative specimens [27]. A component of grade, posttreatment mitotic index was correlated with prognosis, with higher mitotic rate reflecting worse prognosis [28], meaning that lower mitotic counts must have been associated with better prognosis.

The present study has obvious limitations arising from its retrospective nature. Due to the relatively low case numbers in the PST group, there was no chance to analyse the data according to surrogate molecular types of breast cancers or according to the neoadjuvant therapy given, which was not uniform in the series, but generally included docetaxel or paclitaxel for 89% of all patients, and targeted anti-HER2 treatment for HER2 positive tumours.

Conclusions

This is a single-centre study with a large case number reinforcing previous reports on the predominant concordance of grade and its components between CNB and corresponding EXC samples, as well as the tendency to overestimate tubule (gland) formation and underestimate nuclear pleomorphism, mitotic rate and histological grade on CNBs when compared with EXC specimens in non-concordant cases. Many of these tendencies were also seen in cases with pretreatment CNBs and EXC specimens after primary chemotherapy, but importantly, concordance rates were lower; and mitotic scores and histologic grade in EXC specimens were more often lower than higher (in contrast to the primary surgery group). Whether the posttreatment decrease in grade reflects any prognostic value remains to be elucidated.

Acknowledgements

The authors thank the help of Professor Tibor Nyari and Tamas Zombori for their advice in statistics and verification of the results obtained.

Conflicts of interest

The authors have no potential conflicts of interest.

Funding

The study received no funding.

Authors' contributions

ÁF managed the data, performed statistical analyses, drafted the manuscript. GC conceived the study, collected data, drafted and edited the manuscript. Both authors have read and approved the final version of the manuscript.

Ethical consideration

The study was approved by the Human Investigation Review Board, University of Szeged (approval number 90/2021-SZTE-RKEB). The research was conducted ethically, with all study procedures being performed in accordance with the requirements of the World Medical Association's Declaration of Helsinki. An exemption to obtain written informed consent from patients whose material was investigated was part of the ethical approval.

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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

IF (2024/2025): 3,2 (Scimagojr: Q2)

Pathology - Research and Practice 265 (2025) 155732

Contents lists available at ScienceDirect

Pathology - Research and Practice

journal homepage: www.elsevier.com/locate/prp

The prognostic value of histological grade determined after neoadjuvant chemotherapy of breast cancer

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ARTICLE INFO

Keywords:
breast cancer
histological grade
neoadjuvant chemotherapy
post-neoadjuvant chemotherapy grade

ABSTRACT

Histological grade is a validated prognostic factor of breast cancer but may show alterations following neoadjuvant chemotherapy (NACT). Its reporting after NACT is recommended by several guidelines, but evidence of its retained prognostic impact is scarce. Patients treated with NACT followed by surgery and having sufficient residual tumour for the determination of grade were analysed for the survival effects of posttreatment grade (yG). Kaplan-Meier analyses and the log-rank test were applied, followed by the univariable and multivariable Cox proportional hazards models. The cohort comprised 355 patients with known yG, and 320 of them had also a pretreatment grade available. Pretreatment grade changed in 99/320 (31 %) cases following NACT, and downgrading was more common (n=78/320, 24 %) than upgrading (21/320, 7 %). Among 355 breast cancer patients, those with yG3 (poorly differentiated) tumours (n=155) had worse 5-year relapse-free and overall survival estimates than those with yG2 (n=169) or yG1 (n=31) tumours. This was also substantiated by univariable analysis; however, yG lost its significance in the multivariable model. Post-NACT histological grade has a prognostic impact, but does not seem to be an independent prognosticator in the post-NACT setting; however, these results lend support for its reporting by pathologists after primary systemic treatment.

1. Introduction

Breast cancer is a heterogeneous disease that can be stratified along multiple features. Its prognosis is dependent on several factors, traditional ones, like age of the patient, nodal status, tumour size, histological grade, vascular invasion [1–4], and newer ones which require molecular techniques for assessment, like scores derived from gene expression profiles [3,5]. The differentiation of malignant tumours generally reflects their biological behaviour, and the beginning of cancer grading can be traced back to the end of the 19th century, when David von Hansemann published his *Die mikroskopische Diagnose der bösartigen Geschwülste*, linking mitoses and atypia to disease outcome; this was followed by Broders' milestone papers correlating the differentiation of squamous cell carcinomas with their outcome [6]. A three-tiered histological parameter-based grading system of breast cancers was published by Greenhough in 1925 [6,7], and this can be considered the ancestor of the Bloom and Richardson grading system [8] or of the presently used Nottingham or Elston and Ellis histological grading [9] advocated by most current guidelines on reporting breast carcinomas [10–15], because of its well-established prognostic and ensuing treatment influencing role. The histological grade of breast cancer is derived from scoring gland (tubule) formation, nuclear pleomorphism as well as mitotic activity on a 1- to 3-point scale, adding the relevant ordinal values to get a sum, and classify tumours as well, moderately or poorly differentiated, i.e. belonging to grade I, II or III, (G1, G2, G3) respectively.

Abbreviations: 5FU, 5-fluoro-uracil; BxG, core needle biopsy based histological grade; CNB, core needle biopsy; ER, oestrogen receptor; HER2, human epidermal growth factor receptor-2; HR, hazard ratio; NACT, neoadjuvant chemotherapy; OS, overall survival; PR, progesterone receptor; RFS, recurrence-free survival; yG, posttreatment histological grade, 1–3, referring to grades 1–3.

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https://doi.org/10.1016/j.prp.2024.155732
Received 6 October 2024; Received in revised form 16 November 2024; Accepted 20 November 2024
Available online 22 November 2024

Table 1
Basic clinicopathological characteristics of included patients (NST: breast carcinoma of no special type, IDC: invasive ductal carcinoma, ILC: invasive lobular carcinoma, BxG: grade in the core needle biopsy, ER: oestrogen receptor, PR: progesterone receptor, HER2: human epidermal growth factor receptor-2, NACT: neoadjuvant chemotherapy, yG: grade in the resection specimen after neoadjuvant therapy).

Histological type	n	%
NST (IDC)	298	84
ILC	36	10
others	21	6
BxG		
G1	19	5
G2	109	31
G3	192	54
No data	35	10
ER		
Positive	225	63
Negative	127	36
No data	3	1
PR		
Positive	193	54
Negative	158	45
No data	4	1
HER2		
Positive	52	15
Negative	280	79
Unknown	23	6
Molecular subtypes		
Triple (ER/PR/HER2) negative	88	25
HER2 positive (ER and PR negative)	26	7
HER2 positive (ER or PR positive)	26	7
"Luminal-like" (ER and/or PR positive)	192	54
Unclassifiable (missing data)	23	6
NACT		
Taxane-based	285	80
Anthracycline-based	57	16
Other	8	2
Not specified	5	1
Anti-HER2 treatment (mostly trastuzumab alone, or with pertuzumab) (added)	35	10
Surgery		
Excision	105	30
Mastectomy	250	70
yG		
yG1	31	9
yG2	169	48
yG3	155	43
Lymphovascular invasion		
Positive	100	28
Negative	250	71
No data	5	1
ypT		
ypT1a-b	59	16
ypT1c	81	23
ypT2	127	36
ypT3	50	14
ypT4	10	3
ypTx	28	8
ypN		
ypN0	109	31
ypN0(i+)/1 mi	14	4
ypN1	113	32
ypN2	73	21
ypN3	37	10
ypNx	9	2

Neoadjuvant treatment is often used to downstage locally advanced breast cancers to allow breast-conserving surgery and is also helpful in reflecting the response of the tumour to the given treatment. Histological grade has some role in advocating the type of neoadjuvant chemotherapy (NACT), e.g. G1 tumours with low proliferation are unlikely to benefit from classical chemotherapy. It is also known that the grade assessed on pretreatment core needle biopsies (CNBs) most commonly matches the grade established from excisional specimens and when there is a mismatch, most of the time, CNBs underestimate tumour grade [16,17]. In keeping with this finding, we also demonstrated that the correspondence between biopsy grade (BxG) and grade of residual breast carcinoma after NACT (yG) is somewhat lower, and when there is a discrepancy between the two, the yG is generally lower, as a consequence of decreased proliferation, i.e. lower mitotic scores [17].

Most studies reporting on breast cancer patients treated with primary systemic treatment report the initial prognostic profile of their patients, and pathology-based prognosticators of breast cancer in such instances derive from the pretreatment CNBs. Residual tumours are also required to have their grade assessed. The International Collaboration on Cancer Reporting lists this feature as a core element, i.e. an obligatory part of the histopathology report [18], but little is known about yG as a prognostic factor. In the present work, we looked at the prognostic role of histological yG in residual tumours following NACT.

2. Materials and methods

The analysis included a retrospectively collected consecutive series of breast cancer patients treated with NACT followed by surgery at the Departments of Oncotherapy and Surgery, Albert Szent-Györgyi Faculty of Medicine, University of Szeged between 1999 and 2018 and at the Bács-Kiskun County Teaching Hospital, Kecskemét, between 2000 and 2018. Exclusion criteria were the lack of the relevant prognostic parameters (including lack of sufficient residual tumour or poor fixation to determine yG), detection of distant metastasis within 6 months from diagnosis, or lack of follow-up data from the patients' digital charts.

The clinicopathological parameters, namely gender, age, histological diagnosis/histological type [12] from the CNB, BxG of the tumour, type of NACT, type of surgery, completeness of resection, biopsy based oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor-2 (HER2) status, presence of vascular invasion, yG, ypT and ypN categories, finally follow-up data as overall survival (OS) and recurrence-free survival (RFS) were collected from medical charts; these were defined as time from the initiation of NACT to the last follow up or event. There were many chemotherapy regimens used at the two sites during this relatively long period, and therefore the type of NACT was broadly classified as taxane-based (mostly combined with an anthracycline; including docetaxel-epirubicin/doxorubicin ± cyclophosphamide, paclitaxel-epirubicin-capecitabine, paclitaxel-cisplatin, consecutive epirubicin/doxorubicin-cyclophosphamide or cyclophosphamide-epirubicin-5FU and docetaxel, mono paclitaxel); anthracycline-based (when anthracyclines were not combined with taxanes; including cyclophosphamide-epirubicin-5FU, epirubicin/doxorubicin-cyclophosphamide) and others (including few instances of cyclophosphamide-methotrexate-5FU or mitoxantrone-methotrexate ± Mitomycin-C). Trastuzumab (generally alone, on occasion with pertuzumab) was added to NACT in 35/52 HER2-positive cases (Table 1). All patients had regular follow-ups. The follow-up period ended in December 2023.

The proportion of grade changes on NACT was evaluated with contingency tables and the chi-square test.

To evaluate the impact of histological grade on prognosis, Kaplan-Meier analyses and the log-rank test were applied. For further investigations, the univariable Cox proportional hazards model was applied. Factors found significant in the univariable analyses were entered into the multivariable Cox proportional hazards model. Statistical models were fitted using SPSS Statistics V 23.0 software (Armonk, USA). The study was approved by the institutional ethical committee of the Albert Szent-Györgyi Clinical Centre of the University of Szeged.

3. Results

Altogether 355 patients diagnosed with breast cancer, receiving NACT and having residual invasive carcinoma on excision were included. All but one patient were female and the median age was 53

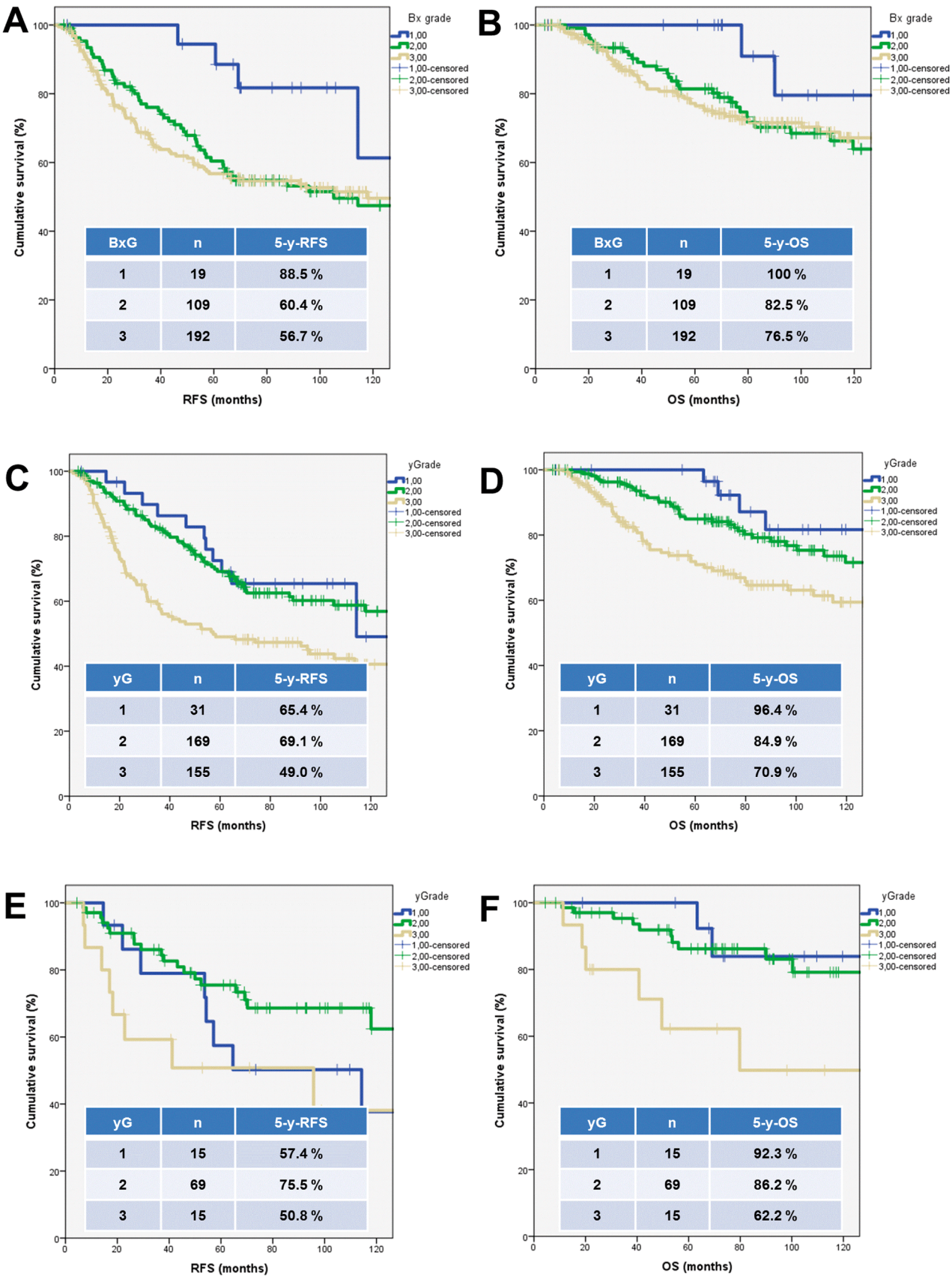

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Fig. 1. The results of the Kaplan-Meier analyses
A and B: Regarding BxG, significant differences were identified among the RFS estimates of BxG 1 vs. 2 (p_{RFS}=0.022) and BxG 1 vs. 3 (p_{RFS}=0.019) but not those of BxG 2 vs. 3 (p_{RFS}=0.356). Furthermore, significant differences were identified between the OS estimates of BxG 1 vs. 3 (p_{OS}=0.05) but not among BxG 1 vs. 2 (p_{OS}=0.075) and BxG 2 vs. 3 (p_{OS}=0.518), respectively. C and D: Concerning yG, significant differences were defined among RFS estimates of yG 1 vs. 3 (p_{RFS}=0.010) and yG 2 vs. 3 (p_{RFS}<0.001) but not those of yG 1 vs. 2 (p_{RFS}=0.788). Significant differences were demonstrated among OS estimates of yG 1 vs. 3 (p_{OS}=0.008) and yG 2 vs. 3 (p_{OS}<0.001) but not between yG 1 vs. 2 (p_{OS}=0.185), respectively. E and F: Subgroup analysis of the cases where BxG and yG were different (n=99). Significant differences were found among RFS estimates of yG 2 vs. 3 (p_{RFS}=0.022), but not among yG 1 vs. 2 (p_{RFS}=0.213) and yG 1 vs. 3 (p_{RFS}=0.463). Furthermore, significant differences were found among 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.

Table 2
Grade changes per molecular subtypes.

Type	All/ assessable*	Grade changes (%)**	Grade decrease/increase ***
Triple-negative	88/78	15 (19 %)	9 (0.6) / 6 (0.4)
HER2+ (HR-)	26/23	5 (22 %)	4 (0.8)/1(0.2)
HER2+ (HR+)	26/25	17 (68 %)	17 (1.0)/0
HR+ (HER2-)	192/175	61 (35 %)	47 (0.77)/14(0.23)
Unclassified	23/19	1 (5 %)	1 (1.0)/0

HR: hormone receptor, i.e. oestrogen receptor and/or progesterone receptor, HER2: human epidermal growth factor receptor-2
* assessable: having both BxG and yG values;
** percent of assessable cases;
*** proportion of grade changes in parentheses

years (range 26–81 years). Basic clinicopathological characteristics are summarised in Table 1. Most patients had ER-positive breast carcinoma of no special type with BxG 3. Most of them received taxane-based NACT and were treated with mastectomy. Approximately 40 % and 70 % of patients were diagnosed with the ypT1 and ypN1–3 categories, respectively. Regarding yG, the grade 1 category was barely identified; whereas the frequency of the grade 2 and 3 categories was almost similar. The median follow-up was 73 months (range: 3.5–236 months); 86 patients died, and 155 experienced disease recurrence.

As the Kaplan-Meier analyses of Fig. 1 demonstrate (Fig. 1 A-D), several significant differences in RFS and OS estimates were found among different comparisons of BxG and yG categories. In general, the higher grade categories of BxG and yG were related to more unfavourable prognoses regarding RFS and OS estimates, respectively. Of the 320 tumours with both BxG and yG data available, the histological grade did not change in 221 (69 %), and there was an increase in grade in 21 (7 %) and a decrease in 78 (24 %) cases. Table 2 displays the distribution of grade changes according to molecular subtypes of breast cancers. As shown in the table, hormone receptor (ER and/or PR) and HER2-positive tumours had the highest rate of grade change on NACT among classifiable cases (chi-square: 22.26, degrees of freedom: 3, p<0.0001), followed by hormone receptor-positive and HER2-negative cases. When these luminal-like tumours were lumped together, their proportion of grade change was still significantly higher than that of triple-negative cancers or HER2-positive hormone receptor-negative cancers (Table 2, chi-square: 11.32, degrees of freedom: 2, p=0.004).

In the subgroup analysis of cases with different BxG and yG (Fig. 1 E-F), the higher categories of yG had an adverse impact on both RFS and OS estimates.

Table 3 displays the results of univariable and multivariable Cox proportional hazards models. Concerning OS, in the univariable analysis, significant adverse prognostic parameters were older age, lack of ER and PR expressions, presence of lymphovascular invasion, incomplete resection, and higher ypT, ypN and yG categories. Regarding RFS, in univariable models, poor prognosis was associated with lack of ER and PR expressions, mastectomy, presence of lymphovascular invasion, and higher ypT, ypN, BxG and yG categories.

In multivariable analysis, regarding 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 (Table 3), respectively.

The univariable subgroup analysis of cases with different BxG and yG categories (data not shown) demonstrated that older age (HR_{OS}: 5.83, 95 % CI: 1.69–20.06, p_{OS}=0.005) and higher yG (HR_{OS}: 5.13 95 % CI: 1.03–25.88 p_{OS}=0.045) category had an adverse impact on prognosis. None of them proved to be independent in multivariable analysis.

The results of the Kaplan Meier subgroup analyses focusing on the prognostic impact of yG and molecular subtypes are displayed in Supplementary Figures 1 and 2. Among hormone receptor (ER and/or PR) positive HER2- 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. Discussion

Histological grade is a well-established traditional prognostic factor of breast cancer either alone [9,12] or in combination with other prognosticators [19–22]. A higher BxG (i.e. G3) is associated with pCR and its effect is independent on the basis of multivariable analysis [23]. Although most of the time, BxG matches the grade established from surgical excision specimens, there is a tendency to underestimate the grade in CNBs. NACT has effects on mitotic activity and nuclear pleomorphism, reducing the first and potentially increasing the second (Fig. 2). Our previous findings suggest that changes in mitotic scores from CNBs to excision specimens (where a reduction was more prevalent after NACT) were significantly different from those seen in the non-NACT setting (where an increase was most common); but no significant differences were demonstrated in nuclear pleomorphism scores [17]. 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 [17]. Whether this change is associated with increased prognosis is not well known.

Although several recommendations prescribe the reporting of histological grade in the post-neoadjuvant setting [18,24,25], a survey among 23 US academic pathologists with breast pathology experience suggested that grade was not reported by 6/23 (26 %); their added comments included the lack of evidence on correlation of yG and prognosis in the post-NACT setting and the drop of mitotic scores with potential senseless downgrading. Those who provided a grade in their reports also made comments and one included that the majority of cases had identical grades but some had a changed grade, with no known evidence (by the responder) of the prognostic impact this could have [26]. Indeed, the prognostic impact of yG has not been widely studied.

The extent of residual disease after NACT has been proven to be of prognostic relevance as reflected by the residual cancer burden (RCB) [27–30], but the addition of Ki67 proliferation index, ER status and yG further improves prognostication [31], suggesting that yG has prognostic importance, too. It should be noted, that the cases analysed in this study have not reached pathological complete regression (pCR), and therefore represent a cohort of patients with worse prognosis than cohorts including pCR patients.

Changes in nuclear atypia after chemotherapy have been described before [32,33], but have not been linked to changes in outcome. On the

Table 3
Results of univariable and multivariable Cox proportional hazards models regarding OS and RFS estimates. (HR: hazard ratio, CI: confidence interval, BxG: grade in the core needle biopsy, ER: oestrogen receptor, PR: progesterone receptor, HER2: human epidermal growth factor receptor-2, NACT: neoadjuvant chemotherapy, yG: grade in the resection specimen after neoadjuvant therapy).

Variable	OS						RFS					
	univariable			multivariable			univariable			multivariable		
	HR	95 %CI	*p*	HR	95 %CI	*p*	HR	95 %CI	*p*	HR	95 %CI	*p*
Age (years)												
<53	reference						reference					
≥53	**1.59**	**1.034–2.45**	**0.034**				1.23	0.90–1.69	0.188			
BxG												
1	reference						reference					
2	2.90	0.69–12.17	0.144				**2.75**	**1.01–7.61**	**0.050**			
3	3.06	0.74–12.60	0.121				**2.86**	**1.05–7.82**	**0.040**			
ER												
positive	reference			reference			reference			reference		
negative	**1.90**	**1.24–2.90**	**0.003**	**2.71**	**1.74–4.20**	**<0.001**	**1.70**	**1.24–2.34**	**0.001**	**1.72**	**1.14–2.59**	**0.009**
PR												
positive	reference						reference					
negative	**1.71**	**1.12–2.61**	**0.013**				**1.57**	**1.14–2.15**	**0.005**			
HER2												
negative	reference						reference					
positive	1.189	0.90–1.55	0.208				0.97	0.77–1.22	0.81			
NACT												
Taxane	reference						reference					
Anthracyclin	1.05	0.61–1.81	0.864				1.04	0.69–1.57	0.833			
Others	1.61	0.51–5.11	0.421				1.16	0.42–3.14	0.772			
Surgery												
Breast-conserving surgery	reference						reference					
Mastectomy	1.33	0.82–2.11	0.251				**1.63**	**1.13–2.35**	**0.009**			
yG												
1	reference						reference					
2	1.87	0.66–5.27	0.235				1.07	0.57–1.99	0.822			
3	**3.39**	**1.22–9.41**	**0.019**				**1.88**	**1.02–3.45**	**0.041**			
Complete resection												
R0	reference						reference					
R1	**1.94**	**1.13–3.30**	**0.014**				1.34	0.86–2.09	0.192			
Lymphovascular invasion												
Absent	reference			reference			reference			reference		
Present	**2.94**	**1.90–4.51**	**<0.001**	**2.26**	**1.42–3.58**	**0.001**	**2.18**	**1.57–3.02**	**<0.001**	**1.84**	**1.27–2.69**	**0.001**
ypT												
ypT1a	reference						reference					
ypT1b	0.42	0.11–1.59	0.205				**0.27**	**0.07–0.93**	**0.039**			
ypT1c	1.19	0.54–2.59	0.658				1.42	0.77–2.61	0.262			
ypT2	1.17	0.55–2.46	0.676				1.33	0.73–2.38	0.344			
ypT3	1.34	0.58–3.08	0.482				1.61	0.84–3.09	0.150			
ypT4	**4.27**	**1.42–12.83**	**0.009**				**4.2**	**1.59–11.05**	**0.004**			
ypN												
ypN0	reference			reference			reference			reference		
ypNmi	1.11	0.14–8.50	0.919	1.75	0.23–13.39	0.591	0.98	0.23–4.12	0.983	2.26	0.52–9.74	0.274
ypN1a	**3.32**	**1.75–6.28**	**<0.001**	**2.95**	**1.52–5.73**	**0.001**	**2.46**	**1.58–3.84**	**<0.001**	**2.36**	**1.42–3.92**	**0.001**
ypN2	**3.50**	**1.76–6.97**	**<0.001**	**3.03**	**1.46–6.24**	**0.003**	**3.06**	**1.90–4.94**	**<0.001**	**3.33**	**1.93–5.75**	**<0.001**
ypN3	**5.11**	**2.39–10.92**	**<0.001**	**4.29**	**1.88–9.79**	**0.001**	**3.16**	**1.78–5.57**	**<0.001**	**3.10**	**1.61–5.97**	**0.001**

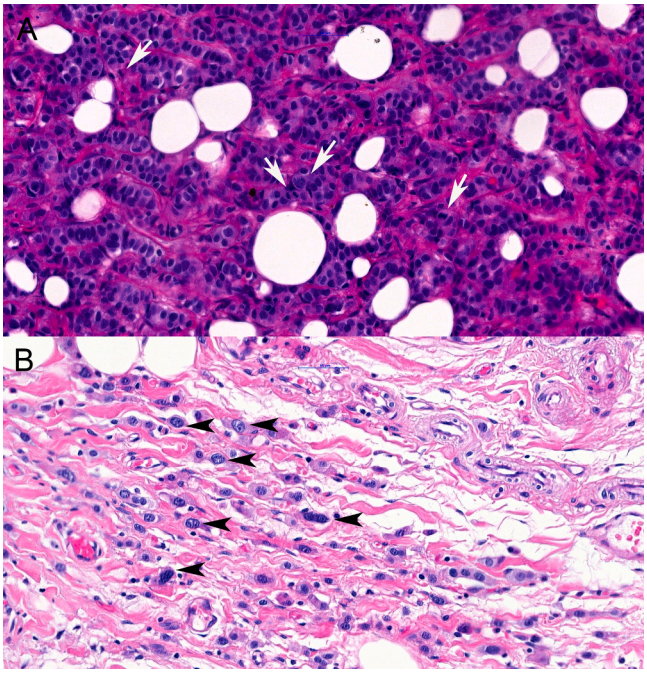

Fig. 2. An example of change in pretreatment grade after neoadjuvant chemotherapy. A: pretreatment BxG3 stemming from tubule formation score 3, nuclear pleomorphism score 2 and mitotic rate score 3. Note mitotic figures highlighted by arrows. B: The patient received NACT consisting of 3 cycles of epirubicin and cyclophosphamide followed by 3 cycles of docetaxel and there was a downgrading occurring, as yG was G2 stemming from tubule formation score 3 (unchanged), nuclear pleomorphism score 3 and mitotic rate score 1. There was cytoreduction, nuclear pleomorphism increased (arrowheads highlight some pleomorphic cells) and there was a significant reduction in mitotic activity. Note, taxanes cause a mitotic arrest, and often this leads to the formation of larger nuclei characterised by polyploidy.

other hand, post-NACT changes in mitotic indices were found to be prognostic [34,35], and this may infer a prognostic value for yG of which this is one of the scored components. Our previous results also point to the fact that whenever there is a change in grade in the pretreatment and posttreatment specimens, this is more likely to be a decrease, and is related to a drop in mitotic activity [17]. In our previous work from a single institution, 24 % (95 % CI: 18–32 %) of breast cancers showed a decrease in grade, which is comparable to this series from 2 institutions, where 24 % (95 % CI: 20–29 %) showed this decrease. An earlier French study documented 34/171 assessable cases (20 %; 95 % CI: 14–27 %) to have a reduction in yG [36]. In the latter study, this improvement in yG was seen more often in responsive tumours and was therefore interpreted as being associated with a benefit from NACT, though survival was not analysed [36].

A previous study of 485 patients treated with anthracycline or anthracycline and taxane-based NACT showed the prognostic impact of both BxG and yG, the impact being greater in the yG setting. The series included 8 % G1, 41.5 % G2 and 50.5 % G3 tumours; with 115 cases (23.7 %) showing pathological complete regression, the remaining 370 residual tumours showed a split of 12 %, 55 % and 33 % between yG1, yG2 and yG3, respectively. The Cox proportional hazards model demonstrated a significant overall and distant disease-free survival disadvantage for yG3 (but not yG1 and yG2) compared to yG0 (reflecting pathological complete regression). However, yG was not assessed separately for cases with residual tumours only [28].

5. Conclusions

Our analysis demonstrates that yG3 is associated with worse RFS and OS than yG1 or yG2, therefore a reduction in grade may be associated with a better prognosis. This is substantiated by the univariable Cox regression analyses, too. However, this role in prognostication does not seem independent, as significance is lost in the multivariable model. Consistent with scarce reports on the prognostic value of yG, these results point to the appropriateness of recommending the determination of histological grade in the post-NACT setting.

Ethical statements

All procedures were performed in compliance with relevant laws and institutional guidelines. This study was approved by the institutional ethical committee of the Albert Szent-Györgyi Clinical Centre of the University of Szeged (91/2021 SZTE-RKEB), the approval includes a waiver of informed consent for this retrospective study; the privacy rights of human subjects was granted by anonymizing data before analysis, as specified in the approval.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

CRediT authorship contribution statement

Zsuzsanna Kahán: Writing – review & editing, Supervision, Methodology. **Tamás Lantos:** Writing – review & editing, Methodology, Formal analysis. **Gábor Cserni:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ádám Ferenczi:** Writing – review & editing, Investigation. **Tamás Zombori:** Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Szintia Almási:** Writing – review & editing, Data curation. **Anita Sejben:** Writing – review & editing, Data curation, Conceptualization. **Renáta Kószó:** Writing – review & editing, Data curation. **Veronika Szelestei:** Writing – review & editing, Data curation.

Declaration of Generative AI and AI-assisted technologies in the writing process

AI use. No AI was involved in the writing of this manucript.

Declaration of Competing Interest

The authors have no conflict of interest. This is also expressed as part of the manuscript.

Acknowledgement

The authors thank the contribution of surgical and medical oncology teams at the two sites for registering the relevant treatment data analysed in this work and are also indebted to the patients involved in the analysis. The publication of this article was made possible with the University of Szeged Open Access Fund, Grant ID: 7387.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.prp.2024.155732.

Data Availability

There have been no computerized data produced in connection with this study. Anonymized data collected and analysed are stored in an Excel file with Hungarian labels and are available from the

corresponding author or first author on reasonable request.

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Glossary

breast cancer: histological grade, neoadjuvant chemotherapy, post-treatment histological grade, prognosis.

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

IF (2024/2025): 3,1 (Scimagojr: Q1)

Virchows Archiv
https://doi.org/10.1007/s00428-025-04263-7

ORIGINAL ARTICLE

Temporality of change in Ki67 labelling following neoadjuvant systemic chemotherapy of breast cancer—the common drop often subsides with time

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Received: 3 July 2025 / Revised: 23 August 2025 / Accepted: 1 September 2025

Abstract

Ki67 immunohistochemistry is widely used in clinical settings to assess tumour proliferation in breast cancer, yet the temporal changes in Ki67 labelling index following neoadjuvant chemotherapy (NACT) are underexplored. Matched core biopsy and surgical excision specimens of invasive breast cancers were collected, and the Ki67 immunostained slides were examined. Ki67 labelling index was determined and statistically evaluated based on the time elapsed between the last cycle of NACT and surgery. Subgroup analyses were carried out based on surrogate molecular subtypes, Residual Cancer Burden (RCB), and European Working Group for Breast Screening Pathology tumour regression (TR) categories. The study included 54 paired breast cancer cases post-NACT. Most tumours were RCB-II ($n = 39$) and TR2b ($n = 20$). The average time from NACT completion to surgery was 40.3 days. Of three methods (including image-analysis and sample-counting), eye-balling estimation provided the best linear regression fit ($p = 0.003$, $R^2 = 0.1548$). Subgroup analysis showed reduced proliferation suppression over time in HER2-positive, triple-negative, and luminal B-like tumours, as well as RCB-II, RCB-III, TR2b, TR2c, and TR3 tumour regression categories. Conversely, luminal A-like tumours, RCB-I, and TR2a categories showed an opposite trend, though limited by small sample size. Multiple studies highlight the prognostic value of Ki67 labelling index post-NACT, with pre- and post-treatment values and change between them serving as independent prognosticators, but our data suggest that the latter two may be time-dependent after the completion of NACT. The proliferation-reducing effect of NACT diminishes over time, particularly in non-luminal A-like tumours and those with poorer treatment responses.

Keywords Ki67 · Breast cancer · Temporality · Neoadjuvant chemotherapy · Post-neoadjuvant

Introduction

Ki67 is a nuclear protein expressed during the G1, S, G2, and M phases of cell division, but not in the resting state (G0); thus, its demonstration by immunohistochemistry (IHC) has been widely used to reflect tumour proliferation in breast cancer [1]. However, Ki67 also raises the problem of interobserver and intraobserver variability and suboptimal reproducibility [2–4].

The prognostic value of the Ki67 labelling index (LI) in breast cancer has been widely studied and a meta-analysis found preoperative (CNB-Ki67) and postneoadjuvant post-operative Ki67 LI (yKi67), as well as the change in Ki67 values from biopsy to postneoadjuvant surgical specimen (ΔKi67) to be independent prognosticators for both overall survival (OS) and recurrence-free survival (RFS) [5]. More precisely, prior research has found CNB-Ki67 LI to be a marker of worse prognosis and also a strong predictor of pathological complete response (pCR) and higher values forecasting a better response to chemotherapy [6–12]. For yKi67, higher values carry a worse prognosis and mark an increased risk of early local and distant relapse [5, 11, 13–15].

Ádám Ferenczi and Tamás Lantos contributed equally to this work.

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Published online: 13 November 2025

Despite both CNB-Ki67 and yKi67 being well documented as prognostic factors in breast cancer by multiple authors, the trend of change in Ki67 over time remains underexamined and less documented. In this study, we aimed to examine the temporality of changes in Ki67 LI values from core biopsies (CNB) to surgical excision specimens following neoadjuvant chemotherapy (EXC) in invasive breast cancers.

Materials and methods

Invasive breast cancers treated with primary systemic chemotherapy with both pretreatment CNB and posttreatment EXC specimens diagnosed at the Bács-Kiskun County Teaching Hospital between 2009 and 2024 were selected. To be included in the study, the cases had to have both CNB and EXC specimens available with an assessable amount of both primary and residual invasive breast carcinoma to determine the grade (at least 2 mm^2 area of the tumour), and Ki67 IHC. Cases with technically suboptimal staining, cut-out material, and only megaslides available with residual tumours were excluded, as well as cases from patients who also received endocrine treatment following the termination of chemotherapy and before surgery. For the remaining cases, the following data were extracted from the histology reports and patients' digital charts: age, tumour type and grade, oestrogen and progesterone receptor (ER, PR) status (+: positive or -: negative), human epidermal growth factor receptor-2 (HER2) status, Ki67 IHC results as original, eyeballing based estimation of the percentage of stained tumour cells (EB-EST), scores for tubule formation, nuclear pleomorphism and mitotic count according to the Nottingham grading system [16], mitotic index (MI: number of mitoses per 10 high power fields corresponding to 2 mm^2), furthermore exact dates of surgery and the last cycle of neoadjuvant chemotherapy (NACT) before surgery, type of neoadjuvant treatment and response to neoadjuvant therapy. Grade and proliferation-related data were obtained for both the CNB and EXC specimens. For the EB-EST method, not only a guess of stained tumour cell percentage was used, but most of the time, areas of roughly 100 cells were virtually evaluated for the proportion of Ki67 positive cells, and the estimation was made using the distribution of such areas and their labelling proportions.

Besides the relatively subjective estimation, the proportion of Ki67-stained tumour cells was also determined using 2 more objective methods. Corresponding slides were collected from the archives and scanned for each case with a Pannoramic 250 scanner (3DHistech, Budapest, Hungary). Three images were taken from each slide with the SlideViewer software (3DHistech, Budapest, Hungary) at ×30 magnification, aiming to represent the tumour as well as possible; in cases with homogenous staining density, 3 random areas were photographed; for cases with heterogeneous Ki67 expression, we aimed to take 2 images reflecting the dominant density and one representing the minority, to allow some weighting for the average. We paid attention not to include pictures containing in situ (DCIS) components. These digital images were then the basis of further analyses.

One method (IR) used the ImmunoRatio 1.0c plugin [17] with the Fiji imagej.net image processing package, which determines positive and negative nuclei by analysing the image according to brown (immunostained, i.e., positive) and blue (haematoxylin stained, i.e., negative) colour thresholds. The source image scale (4.0 pixel(s)/μm) and correction equation ($y = ax\text{^}3 + bx\text{^}2 + cx + d$; $a = 6.442$, $b = 0$, $c = 0.611$, $d = 0.43$) values remained as default. The brown and blue colour thresholds were modified at each image to best represent the unique nuclear staining observable on each slide. A limitation of the software observed during analysis was the fact that in cases where the slide was understained or heavily overstained, or the cell density was remarkably high, the software had trouble differentiating single nuclei. This led to a blurring effect, which was unresolvable even with colour threshold adjustments because a decrease in colour sensitivity led to multiple nuclei not being recognised. In these troublesome cases, the colour thresholds were adjusted so that the results best represented all tumour cells. The minimal sensitivity was determined for each digital image to allow all tumour cell nuclei to be recognised, and the blurring effect was minimised to avoid false positive recognition. This limitation was not present on slides where the contrast between nuclear and cytoplasmic or background staining was more intense. During the analysis of slides that contained large foci of connective tissue or inflammatory cell infiltrate, the image was masked to only contain tumour cells, thus removing all other nuclei from the recognition analysis.

The third method of Ki67 quantification was a grid counting (GRID) following the description of Vörös et al. [18]: 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, was spread over the images. The nuclei which were intersected by these lines were then counted manually, and the ratio of positive to all nuclei was calculated.

To characterise the cases, the Ki67 staining percentages were used. For the EB-EST method, the whole slide and the whole available material were considered for pretreatment CNBs, and a single tumour slide was Ki67 stained as representing the posttreatment EXC specimen; the whole slide examination was the basis for the estimation of the average labelling. For the IR and GRID methods, the average of the quantification of the 3 representative images was taken. By the end, for each tumour, there were three pairs of CNB and

EXC Ki67 labelling values, to follow the time-dependency of changes in proliferation after the last cycle of NACT. Data were collected and managed in Microsoft Excel.

Cases were grouped for subset analyses. For molecular subtypes, we used an IHC-based approach distinguishing between HER2-positive (scored either 3 + or being amplified on in situ hybridisation [19]), triple-negative (TN: ER-PR-HER2-), and ER+HER2-, with a 1% staining required for ER+ or PR+ [20]. One single case of ER-PR+HER2- was lumped with the ER+HER2- cases. The response was categorised according to the Residual Cancer Burden (RCB) main classes I to III [21] which also includes parameters of the lymph nodes after NACT, and alternately, according to tumour response (TR) categories proposed by the European Working Group for Breast Screening Pathology: TR2a with < 10% residual cancer, TR2b with 10 to 50% residual cancer, TR2c with > 50% residual cancer but signs of regression, and TR3 for cases with no regression [22]. Examples of pre- and post-NACT cases with different TR categories are shown in Supplementary Fig. 1.

Linear regression analyses were performed to evaluate the effect of the time elapsed between the last cycle of chemotherapy and surgery (independent variable) on the change between starting and end values (i.e., the difference between EXC and CNB values, in this order; dependent variable). The model fits were quantified by the R-squared statistic (coefficient of determination; R^2). Subgroup analyses were carried out by surrogate molecular subtypes, RCB, and EWGBSP regression categories, respectively.

Agreement between two measurement methods was assessed using Bland–Altman analysis. For each paired observation, the difference between the two methods was plotted against their mean. The mean difference (bias) and the 95% limits of agreement (mean difference ± 1.96 × standard deviation of the differences) were calculated to quantify the agreement. A linear regression of the differences in the mean values was performed to check for proportional bias. All statistical analyses were conducted using R (version 4.4.1). *p*-values of less than 0.05 were considered to be statistically significant.

Results

After all exclusions, a total of 54 paired cases were included in our study. Clinical data of the patients and tumours are summarized in Supplementary Table 1. The most common surrogate molecular subtype observed was luminal B-like ($n = 22$), followed by TN ($n = 16$), HER2-positive ($n = 12$), and finally luminal A-like ($n = 4$). The majority of the tumours showed regression grades of RCB-II ($n = 39$) and TR2b ($n = 20$). The average time between the last cycle of NACT and surgery was 40.3 days (range: 8–82).

PST in 52/54 cases consisted of a taxane-containing regimen, most often involving the sequential administration of epirubicin and docetaxel, but some patients received a taxane plus platinum regimen or other taxane-containing regimens. As for the two remaining cases, one consisted of 3 cycles of epirubicin (as the patient declined further preoperative chemotherapy), and the other consisted of capecitabine. Ten out of 12 patients with HER2-positive tumours also received 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).

All cases were reported by the same pathologist, or the slides reported by others were reviewed by the same pathologist (GC), to limit interobserver variation. The same microscope was used for scoring mitoses during the period. A representation of the results of the IR method, as well as a representation of the gridlines used for the GRID method, is illustrated in Fig. 1.

Out of all methods, the best goodness of fit for the linear regression model was achieved by the EB-EST method, indicating an upward trend, thus a decreasing change in Ki67 LI over time when all cases were grouped ($p = 0.003$, $R^2 = 0.1548$). The linear regression results and R^2 values for each quantification method are presented in Fig. 2.

In the subgroup analysis based on surrogate molecular subtype (Supplementary Table 2), luminal A-like tumours had the best fit with the linear regression model ($p = 0.125$, $R^2 = 0.766$), indicating an increasing rate of proliferation blocking effect as time passes after the last cycle of NACT, albeit this subgroup contained the fewest number of cases ($n = 4$). HER2-positive ($p = 0.091$, $R^2 = 0.2589$), TN ($p = 0.045$, $R^2 = 0.2563$), and Luminal B-like ($p = 0.039$, $R^2 = 0.1961$) tumours all demonstrated an upward trendline in the linear regression model, indicating that these tumours had a decrease in the proliferation blocking effect of NACT over time. The results of the subgroup analysis based on surrogate molecular subtypes are demonstrated in Fig. 3, alongside the R^2 values.

Subgroup analysis based on RCB regression grade scores indicated discordant trendlines between RCB-I versus RCB-II and RCB-III categories, with the former indicating a downward trend and the latter two having an upward trend, meaning a decrease in mitotic rate reduction with time after NACT. RCB-I and RCB-III categories contained a suboptimal number of cases ($n = 8$ and $n = 7$, respectively), which is a limitation of our study. The best goodness of fit was achieved in the RCB-II group ($p = 0.003$, $R^2 = 0.209$), which also contained the highest number of cases ($n = 39$). The resulting linear regression models with their corresponding R^2 values are presented in Fig. 4.

When analysing the cases according to EWGBSP regression grade categories, the linear regression trendline of TR2a tumours had a downward slope ($p = 0.278$, $R^2 = 0.2819$),

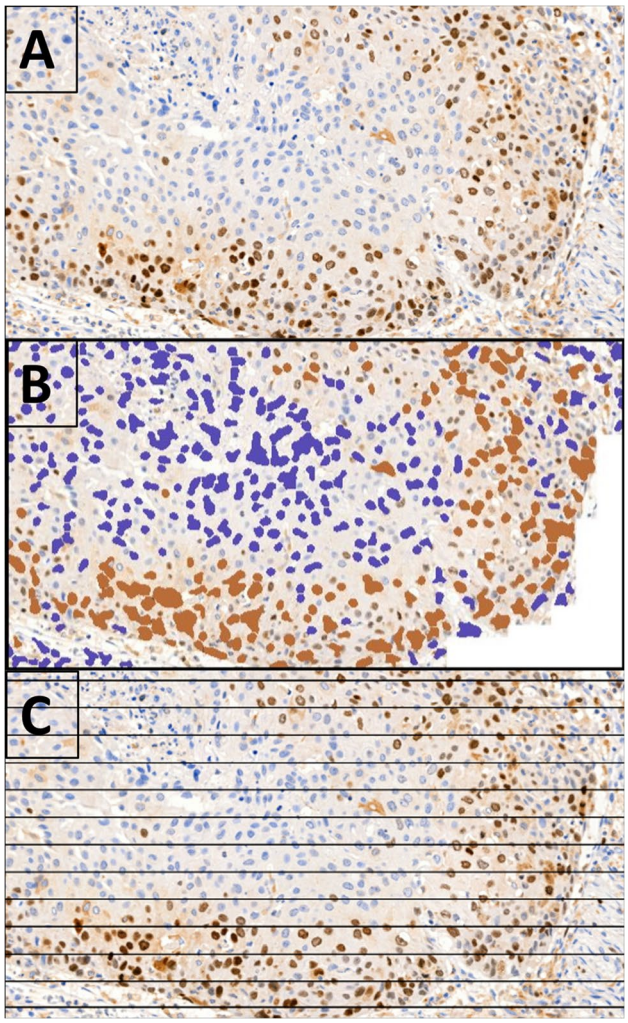

Fig. 1 Illustration of the methods used (Ki67, 30x). **A** Eye-balling estimation (EB-EST) was made after examining the whole slide, looking at areas of about 100 cells and the number of labelled nuclei, and trying to average these; **B** ImmunoRatio (IR); relatively good contrast between the blue and brown colours helps the software accurately analyse the scanned Ki67 stained slide image; and **C** the GRID method utilized gridlines spaced equally on top of the scanned slide image and the intersected nuclei were counted

indicating a sustained effect of chemotherapy, while TR2b ($p = 0.051$, $R^2 = 0.1951$), TR2c ($p = 0.028$, $R^2 = 0.3209$) and TR3 ($p = 0.02$, $R^2 = 0.4019$) groups demonstrated an opposite, upward trend in the change of Ki67 over time (Fig. 5).

Regarding the Ki67 LI values in core biopsies, the Bland–Altman (BA) 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 (Supplementary Fig. 2). However, no significant pattern (that is, trend) in the

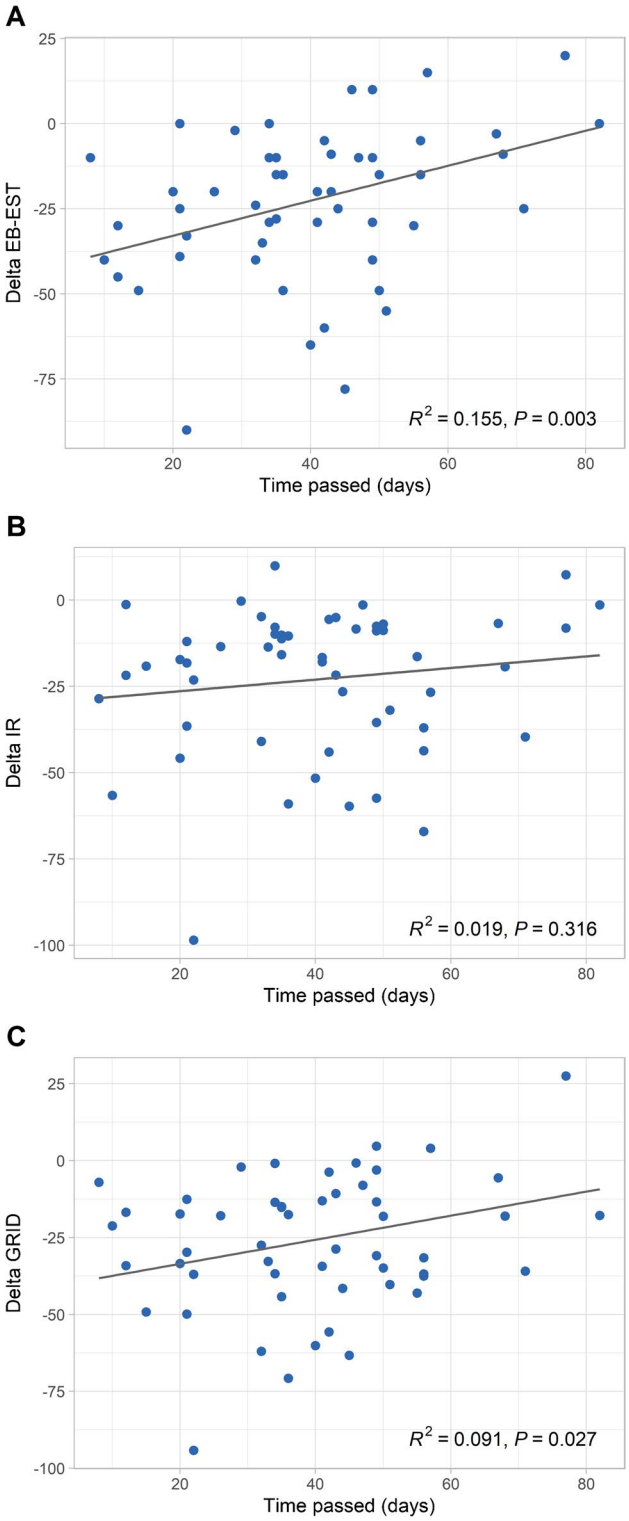

Fig. 2 Scatter plot (each point represents an observation) with the fitted trend (line) and model fit (R-squared) illustrating the relationship between change (delta) in the value of Ki67 labelling index and days passed between last cycle of systemic chemotherapy and surgery for each observation method included in the study such as **A** eye-balling based estimation (EB-EST), **B** ImmunoRatio image processing package (IR), and **C** cell counting with the help of gridlines spread over the scanned slide image (GRID)

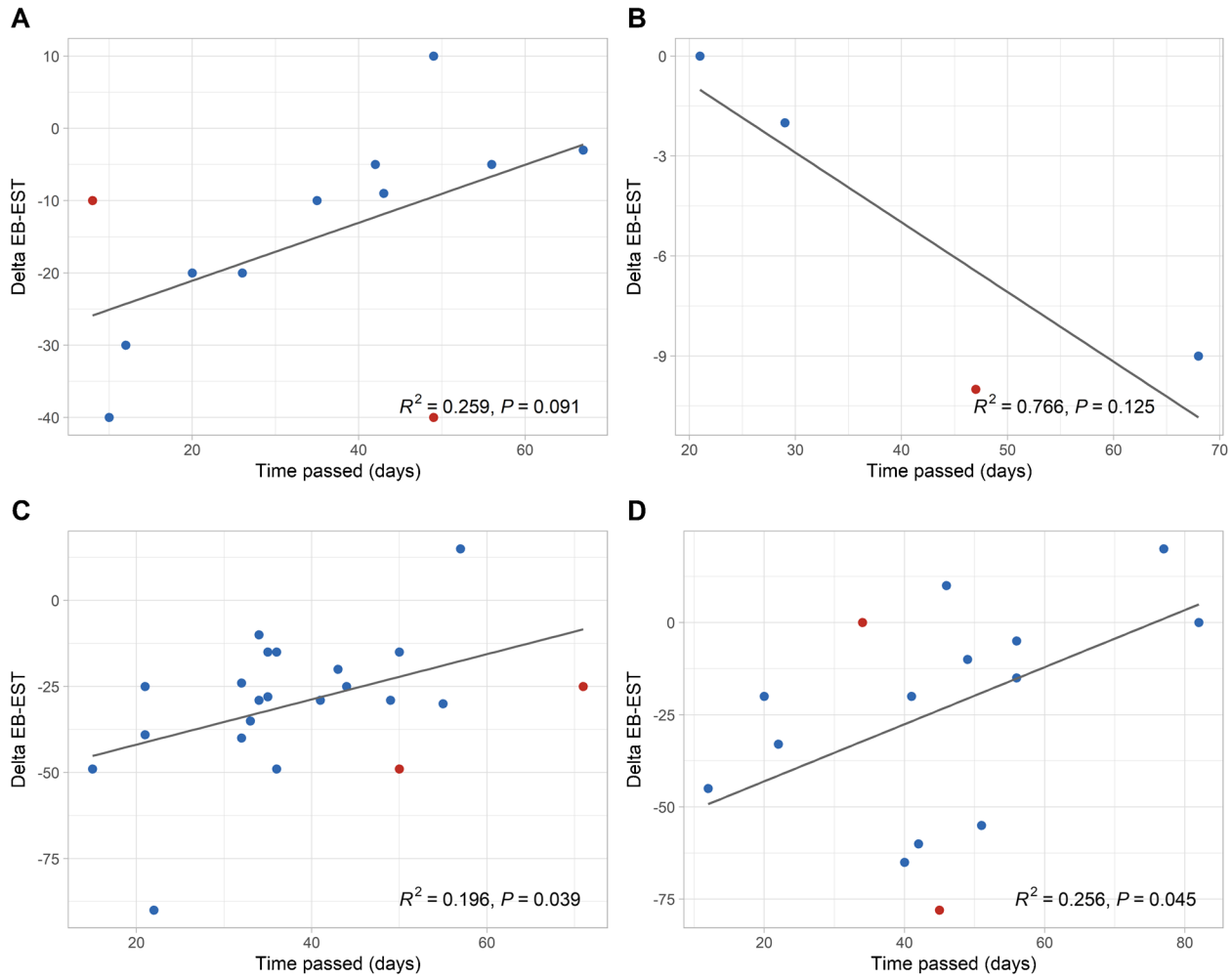

Fig. 3 Scatter plot (each point represents an observation) with the fitted trend (line) and model fit (R-squared) illustrating the relationship between change (delta) in the value of Ki67 labelling index and days passed between last cycle of systemic chemotherapy and surgery for eye-balling based estimation (EB-EST) according to surrogate molecular subtypes (subgroup analysis) such as **A** HER2+, **B** luminal A-like, **C** luminal B-like, and **D** TN tumours. Statistical outliers are indicated in red. Footnote: HER2+, human epidermal growth factor receptor-2-positive breast cancers; TN, triple-negative breast cancers

differences was observed across the range of the methods pairwise ($p = 0.874$ and $p = 0.112$, respectively).

As for the values in excision specimens, the BA 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 range for limits of agreement was −24.2 to + 43.9 and −15.9 to + 14.1, respectively (Supplementary Fig. 3). Additionally, significant increasing trends in the differences were detected across the range of methods ($p < 0.001$ and $p = 0.01$, 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/GRID as values increase (Supplementary Fig. 4).

Discussion

In recent years, the significance of NACT has been increasing, as it allows for a more conservative surgical approach by down-sizing, as well as monitoring the tumour response to the chemotherapeutic agents and eliminating micrometastases in high-risk early or locally advanced breast cancers [6]. Pathological complete response (pCR) after NACT has been the focus of multiple studies and has proven to be a good predictor of survival [23]. It has also been noted that pCR is only achieved in a minority of cases after NACT, approximately 15–20%; thus, it remains of great importance to further study possible predictive factors and prognosticators in residual disease [24].

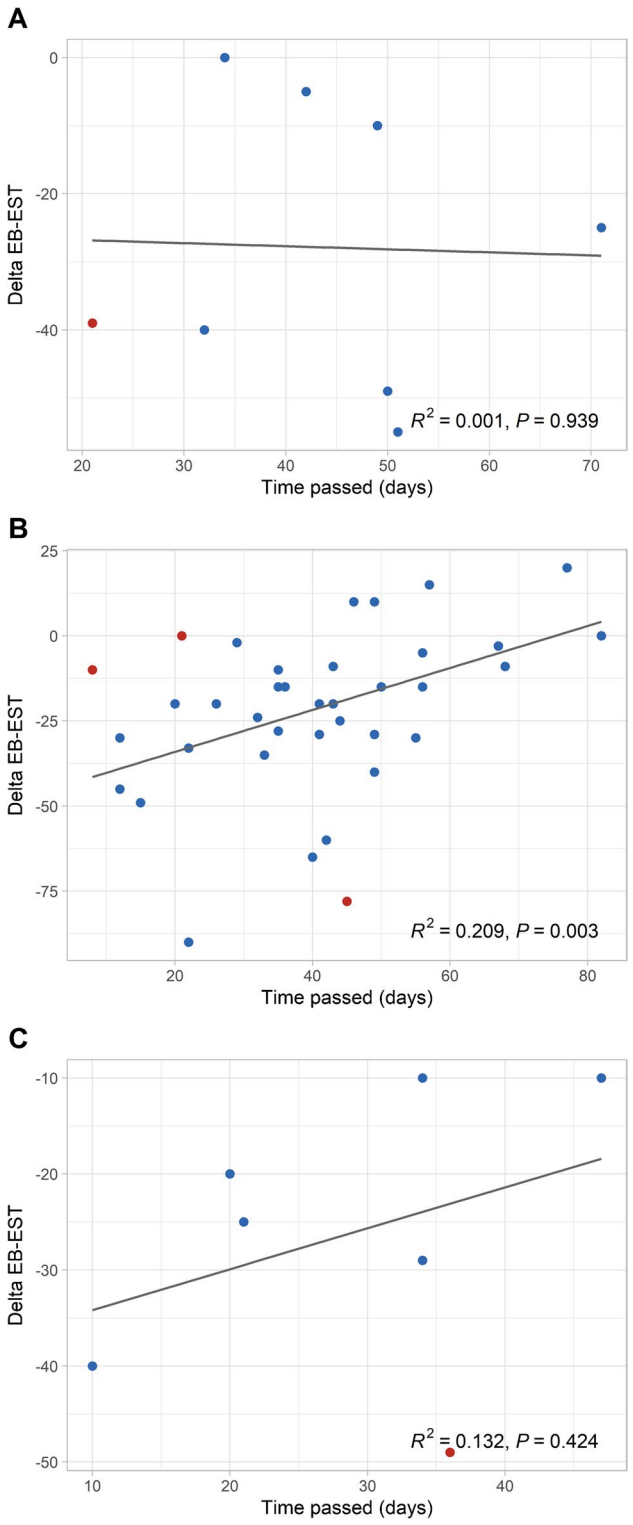

Fig. 4 Scatter plot (each point represents an observation) with the fitted trend (line) and model fit (R-squared) illustrating the relationship between change (delta) in the value of Ki67 labelling index and days passed between the last cycle of systemic chemotherapy and surgery for eye-balling-based estimation (EB-EST) according to RCB tumour regression grading scores (subgroup analysis) such as **A** RCB-I, **B** RCB-II, and **C** RCB-III. Statistical outliers are indicated in red. Footnote: RCB, residual cancer burden; RCB-I, RCB score greater than 0 and less than or equal to 1.36; RCB-II, RCB score greater than 1.36 and less than or equal to 3.28; RCB-III, RCB score greater than 3.28

The effectiveness of NACT is highly dependent on the biological behaviour of breast cancer, supported by the fact that it is mostly given to patients with poorly differentiated (G3), highly proliferative tumours, because the proliferation-blocking effects of NACT may be more beneficial in these cases. In our previous work, we were able to reproduce and statistically describe the changes observed in histological grade and its component scores for tubule formation, nuclear pleomorphism, and mitotic rate, which have been reported by other authors as well [25]. Specifically, if there was a change from CNB to EXC, the grade tended to decrease rather than increase as a result of mitotic rate reduction in the group treated with NACT [26]. Being able to monitor the response of a tumour to this proliferation-blocking effect of NACT might help identify patients who might derive less benefit from the given therapies.

Dynamic, biomarker-based adjustment therapy regimens may offer a way to separate the therapeutic pathways of well-responders from non-responders to reach the best-personalised outcome, avoiding under- or overtreatment. In hormone receptor (HR)-positive, HER2-negative intermediate risk tumours, this question has been assessed by the WSG-ADAPT-HR+/HER2- sub-trial, based on Ki67 response. Treatment was tailored based on a second, interim biopsy sample taken after 3 weeks of induction endocrine therapy and its Ki67 LI value. Patients meeting the threshold ≤ 10% Ki67 LI continued to receive endocrine therapy only, while those with Ki67 LI > 10% also received chemotherapy. The trial concluded that Ki67 from interim biopsies was valuable to assess response to therapy. Similarly, the POETIC trial concluded that Ki67 was useful for the differentiation of well-responder and non-responder groups, yKi67 LI ≤ 10% in EXC specimens from surgery 2 weeks after randomization was prognostic, and adjuvant endocrine therapy alone was sufficient for responders, whereas non-responders might benefit from further adjuvant treatments [27–30].

Adjusting NACT regimens based on tumour proliferation change has been studied by Mukai et al., who monitored the effects of NACT by assessing Ki67 from interim biopsy specimens in HER2-positive tumours and divided their patients into Ki67 responder and non-responder groups. They concluded that switching the original weekly paclitaxel and trastuzumab therapy regimen to epirubicin combined with cyclophosphamide and trastuzumab produced worse pCR rates in non-responders than the pCR rates of patients maintained on the original NACT [31]. We are not aware of other studies investigating the adjustment of NACT according to Ki67 response; therefore, further studies of possible therapeutic agents might be needed to reach a better conclusion on Ki67 adjustment of treatment in this subset of patients.

According to the 2015 St. Gallen consensus conference, the main IHC surrogate subtypes of breast cancer

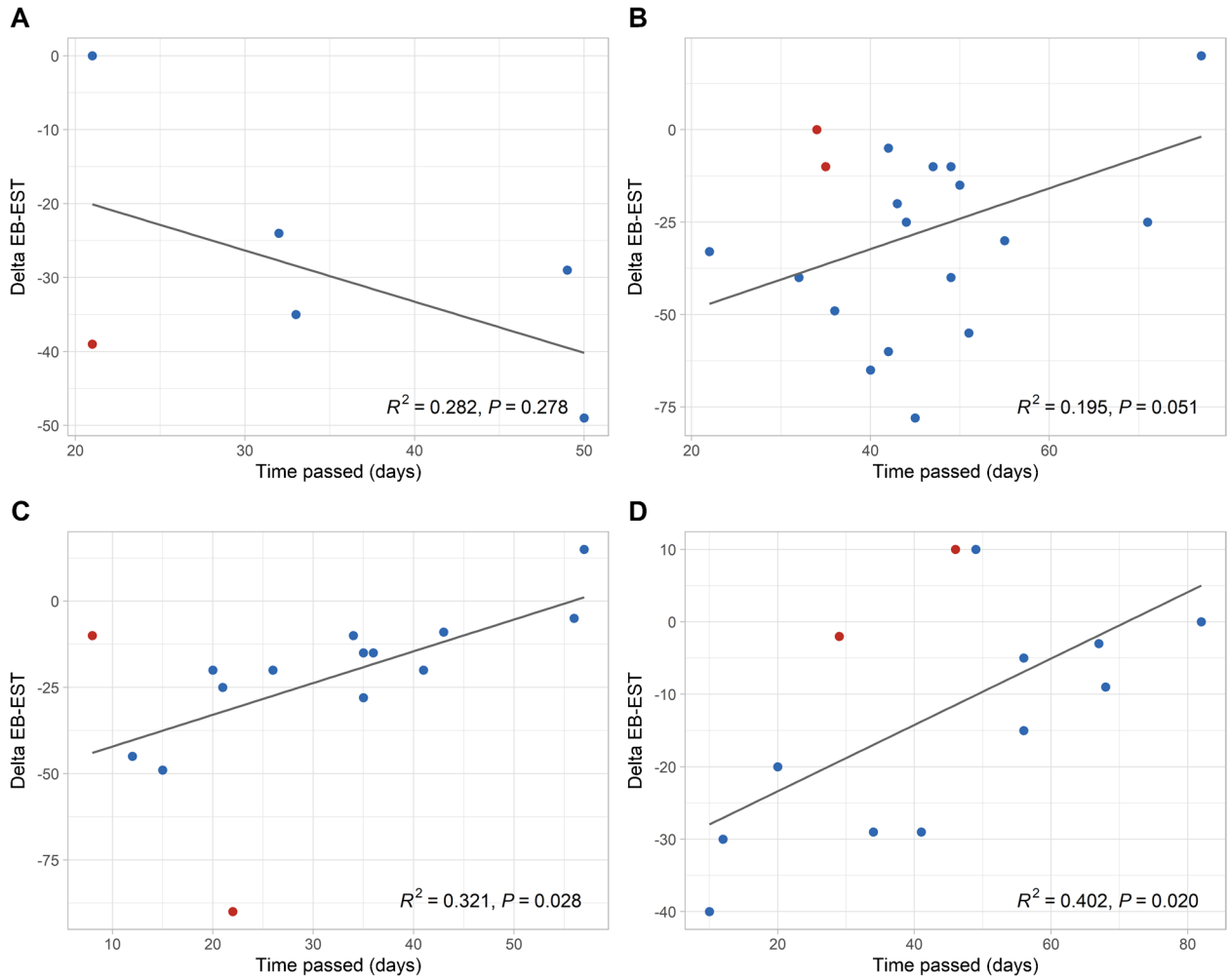

Fig. 5 Scatter plot (each point represents an observation) with the fitted trend (line) and model fit (R-squared) illustrating the relationship between change (delta) in the value of Ki67 labelling index and days passed between the last cycle of systemic chemotherapy and surgery for eye-balling based estimation (EB-EST) according to EWGBSP tumour regression scores (subgroup analysis) such as **A**: TR2a, **B**: TR2b, **C**: TR2c and **D**: TR3. Statistical outliers are indicated in red EWGBSP: European Working Group for Breast Cancer Screening, TR2a: < 10% residual cancer, TR2b: 10-50% residual cancer, TR2c: > 50% residual cancer but with signs of regression, TR3: no signs of regression

are triple-negative breast cancer (TNBC), HR+HER2+, HR-HER2+, and the HR+HER2- groups, the latter consisting of luminal A-like and luminal B-like tumours, among which Ki67 LI is an important differentiating factor [32]. While there is no consensus on the cut-off value between low and high Ki67, the most commonly observed values were between 15 and 30%, the latter being the threshold for high proliferation currently suggested by the International Ki67 in Breast Cancer Working Group and the latest St Gallen Consensus Conference dealing with this issue, while 20% had been the cut-off for separating luminal A-like tumours from luminal B-like ones [1, 6, 12, 13, 23, 33–37].

Chen et al. reported a 37.5% discordance rate in Ki67 LI between CNB and post-NACT EXC specimens using a > 20% cut-off to dichotomise tumours as of high or low proliferation. The survival of patients with pre- and post-treatment Ki67 both ≤ 20% was significantly better than any of the concordantly high or discordant low to high or high to low converted cases [23]. Our previous study comparing cases treated with primary surgery or NACT suggests that based on mitotic counts, discordantly scored biopsies tend to underestimate proliferation in general (primary surgery cases), but in NACT cases, proliferation in core biopsies is more often higher than in post-NACT specimens [26], and this result is in keeping with the results of Chen et al. [23]. Furthermore, we also demonstrated that post-NACT histological grade has also prognostic impact [38].

While ΔKi67 was also recognised as prognostic, yKi67 was 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 [5, 11, 13–15]. Many authors seem to agree on the prognostic value of yKi67 LI [5, 13, 39, 40].

A comprehensive meta-analysis was done by Li et al., examining preoperative and postoperative Ki67 as well as ΔKi67 from biopsy to excision in terms of OS and disease-free survival (DFS). Their study concluded that all three of these markers are independent prognostic factors of both OS and DFS, this finding being backed by other studies since then [12, 24, 41]. Furthermore, yKi67 proved to be a stronger predictor of DFS than of OS [5, 34, 35].

We may conclude from previous studies that Ki67 LI assessed both from CNB and EXC specimens carries important information and has an impact on prognosis. Not only the starting and end values (i.e., CNB and EXC specimen values) but also the degree of change between the two can alter the expected prognosis, and yet, the temporality and trend of this 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 molecular subtype using the 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 EWG-BSP TR2a), all underrepresented in the cohort. The data are purely descriptive and seem to reflect a dynamism in the proliferation-reducing effect of NACT with time from termination in most breast cancers, with the potential exception of the luminal A-like tumours (unlikely to receive NACT) or the ones with good response to treatment, where the effect may be more permanent. 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 (Fig. 2); we have finally selected EB-EST based on the best goodness-of-fit and its widespread use in clinical practice. The absolute values of Ki67 differed by method, and probably artificial intelligence-based quantification would have been the most robust support for substantiating our findings, but due to the current unavailability of this technology in our hands, we sought to demonstrate the similarities in the results using different methods of assessment.

As a limitation, the relatively small sample size of this study interferes with the interpretation of the results in the smallest subsets. Eliminating outliers could have improved the goodness-of-fit of the regression models, but low case numbers have hampered this.

Conclusions

In conclusion, 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.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00428-025-04263-7.

Author contributions All authors made substantial contributions to the conception or design of the work (ÁF, TL, GC); the acquisition (ÁF, GC), analysis (ÁF, TL), interpretation of data (ÁF, TL, GC); drafted the work or revised it critically for important intellectual content, approved the version to be published and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved (ÁF, TL, GC).

Funding Open access funding provided by University of Szeged. The study received funding from the University of Szeged, University Research Fellowship Programme (EKÖP-291). Open access funding provided by the University of Szeged, (Open Access Fund, Grant No. 8021).

Data availability Anonymised data collected and analysed are stored in an Excel file with Hungarian labels and are available from the corresponding author upon reasonable request.

Declarations

Ethics approval All procedures were performed in compliance with relevant laws and institutional guidelines. The study was approved by the Institutional Ethical Committee of the Albert Szent-Györgyi Clinical Center of the University of Szeged (approval number 91/2021-SZTE-RKEB), the approval includes a waiver of informed consent for this retrospective study; the privacy rights of human subjects were granted by anonymizing data before analysis, as specified in the approval.

Conflict of interest The authors declare no competing interests.

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Co-author certification

I, myself as a corresponding author of the following publication(s) declare that the authors have no conflict of interest, and Ádám Ferenczi Ph.D. candidate had significant contribution to the jointly published research(es). The results discussed in his thesis were not used and not intended to be used in any other qualification process for obtaining a PhD degree.

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The publication(s) relevant to the applicant's thesis:

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

IF (2024/2025): 3,2 (Scimagojr: Q2)