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Time-Dependent Prognostic Value of Ki-67 in Early Breast Cancer: Validation of Visual and Hot-Spot Scoring Methods

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ABSTRACT

Ki-67 proliferation status measured by immunohistochemistry has long been discussed as both a prognostic and predictive indicator in breast carcinoma, yet its clinical robustness remains uncertain. In this analysis, Ki-67 was reassessed retrospectively by three independent pathologists using two scoring strategies—a whole-slide visual estimate and a focused quantitative count at the tumour edge—in a cohort of 411 early breast cancer patients followed for a median of 26.8 years. Agreement among observers was excellent for both scoring approaches. Recurrence risk linked to Ki-67 varied over time: individuals with high proliferation (Ki-67 ≥ 30%) initially showed greater recurrence rates, but after roughly 4.5 years, the lower proliferation category exhibited increased late risk. Among estrogen receptor (ER)-positive cases, the intermediate Ki-67 group behaved similarly to the high-proliferation category early on, then diverged to resemble the low-proliferation outcomes later. ER-positive pN0-1 patients with mid-range Ki-67 who received endocrine therapy alone achieved outcomes comparable to those treated with chemotherapy. A threshold near 20% appeared optimal for separating low- and high-proliferation groups. Overall, straightforward visual scoring of Ki-67 on full slides proved reliable for clinical use, and Ki-67 was reaffirmed as a key prognostic and predictive biomarker.

Keywords: Breast cancer, Immunohistochemistry, Ki-67, MIB-1, S-phase fraction, Proliferation

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Introduction

Breast cancer remains the most frequently diagnosed malignancy in women globally, representing 24% of all cancers and standing as the primary cause of female cancer mortality [1]. In Slovenia, the crude incidence from 2016–2020 reached 142.2 per 100, 000 women, with a mortality of 42.1 per 100, 000 [2].

Adjuvant therapy is used to lower the risk of relapse and death. Multiple tools contribute to recurrence-risk estimation. Beyond traditional prognostic attributes—tumour size, nodal status, histologic grade, ER expression, and HER2 status—tumour proliferation adds uniquely informative biological insight. Cell division is a core hallmark of malignancy [3]. Proliferative activity can be assessed by mitotic counting, S-phase estimation via flow cytometry (S-phase fraction; SPF), immunohistochemistry for proliferation markers such as Ki-67, and, more recently, by genomic assays that incorporate numerous proliferation-related genes (Oncotype DX, Genomic Grade Index, PAM50) [4-7]. Despite these options, IHC detection of Ki-67 (with the MIB-1 clone) remains the most commonly used method for routine evaluation of tumour proliferation and for predicting chemotherapy benefit.

Ki-67 is a nuclear antigen present in all active phases of the cell cycle but absent in G0, making it easily detectable by IHC [5]. Numerous studies have examined Ki-67 as both a prognostic and a predictive metric [8-11]. Following the discovery of molecular subtypes based on gene expression, Ki-67 became decisive for distinguishing luminal A from luminal B breast cancers using a <14% cut-off [9]. Luminal B tumours show worse outcomes than luminal A [9]. Surrogate IHC classifications were endorsed by the 2011 St. Gallen guidelines [12]. Later, most panel members supported ≥20% as the threshold for “high” Ki-67, independent of molecular subtype [13]. A meta-analysis by de Azambuja and colleagues demonstrated that elevated Ki-67 (cut-off 5–30%) predicted reduced

disease-free survival (DFS) and overall survival (OS) broadly and in both node-negative and node-positive groups [14]. Additionally, Yerushalmi *et al*. reported that high Ki-67 predicted neoadjuvant chemotherapy response, while low Ki-67 suggested greater endocrine sensitivity [8].

Historically, SPF measured by flow cytometry provided a quantitative and efficient means to evaluate proliferation and DNA ploidy. In a previous 770-patient cohort from our institution, 43% had diploid tumours, and the median SPF was 6.7%. High SPF (≥6.7%) independently predicted poorer DFS and OS [15], in line with a review by Colloza *et al*. [16]. Many studies documented a strong correlation between SPF and Ki-67 [17-23], though some failed to show this relationship [24, 25].

Even though Ki-67 correlates strongly with outcomes and therapeutic benefit, it has not been universally adopted due to concerns about reproducibility. To reduce variability, the International Ki-67 in Breast Cancer Working Group (IKWG) issued recommendations in 2011 [26], updated in 2021 [27]. Two IKWG reproducibility studies showed high intra-laboratory reliability but only moderate between-laboratory concordance [28, 29]. Consequently, uncertainty persists regarding clinically robust cutoffs for prognosis and therapeutic decision-making [30]. The updated guidelines did not endorse a single threshold but proposed ≤5% for low and ≥30% for high proliferation [27, 30]. ESMO recommendations consider Ki-67 ≤ 10% clearly low and ≥30% high [31], while ASCO and NCCN currently do not include Ki-67 in routine treatment planning.

Our pathology department has extensive experience with both Ki-67 and SPF. In this long-term retrospective study, spanning nearly three decades, proliferative activity was assessed using both methods. We analyzed inter-observer consistency for each Ki-67 scoring technique, compared the two Ki-67 approaches with SPF, and evaluated their prognostic and predictive value in the full cohort and in ER-positive patients. Our working premise was that both Ki-67 methods would demonstrate strong observer agreement, correlate reliably with SPF, and serve as independent indicators of prognosis and treatment responsiveness.

Materials and Methods

Patients and treatment

This retrospective cohort consisted of individuals diagnosed with early-stage breast cancer at the Institute of Oncology Ljubljana, Slovenia, between 1992 and 1997. Clinical information—including tumour dimensions, histologic subtype, grade, lymphovascular invasion status, axillary node involvement, ER expression, and therapeutic management—was extracted from existing medical documentation. At that time, ER status was measured biochemically [32], using 10 fmol/mg protein as the threshold for a positive result. HER2 information was not available because it was not routinely assessed during that period.

Eligibility required patients to be ≥18 years old, have disease classified as stage IA–IIIB, undergo primary surgery, and have both SPF data and retained formalin-fixed paraffin-embedded (FFPE) samples. Individuals with in situ carcinoma, distant metastases, preoperative systemic treatment, absence of usable FFPE material, or unsuccessful IHC processing were excluded.

Ethical clearance was granted by the Slovenian Medical Ethics Commission (No. 0120-176/2019-4). All study procedures adhered to the Declaration of Helsinki and followed Good Clinical Practice guidelines.

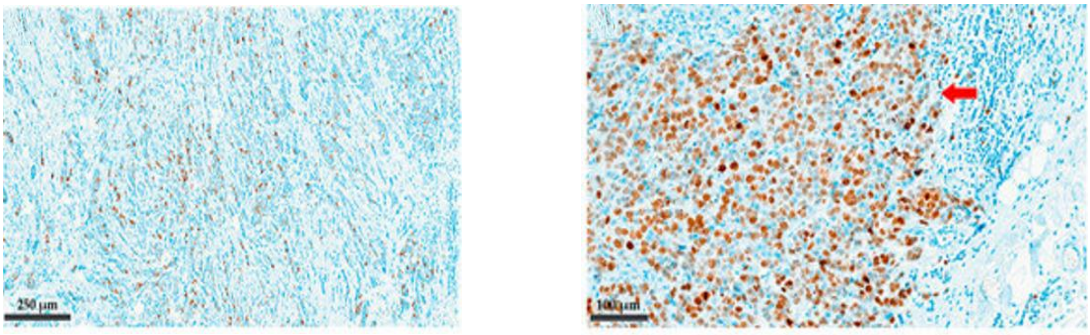
Pathohistological examinations: IHC staining and Ki-67 scoring

Criteria for biomarker study reporting (BRISQ [33] and REMARK [34]) were observed.

IHC analyses were conducted on archival FFPE tumour excisions, fixed in 10% neutral buffered formalin for 8–72 hours. Slides of 2–4 μm thickness were freshly cut and dried at 56 °C for 2 hours, then processed on the automated Ventana BenchMark ULTRA platform (Ventana, Roche Diagnostics, Tucson, AZ, USA). Antigen retrieval was carried out for 88 minutes at 100 °C using Cell Conditioning Solution 1 (cat. No. 950-124). Ki-67 was detected with the monoclonal antibody MIB-1 (Agilent, Santa Clara, CA, USA; cat. No. M7240; dilution 1:200). Incubation proceeded for 60 minutes at 37 °C, followed by visualization using the OptiView DAB IHC Detection Kit (Ventana ROCHE Inc.; cat. No. 760-700). Slides were counterstained with haematoxylin (cat. No. 790-2208). Internal control sections contained normal appendix, cervical epithelium, and three breast cancers representing different proportions of Ki-67–positive cells. The laboratory's Ki-67 protocol has repeatedly achieved high marks in external quality schemes (UK NEQAS, NORDIQC, LabQuality).

Ki-67 staining was evaluated independently by three readers—two breast-pathology specialists (SD, BGK) and one pathology trainee (TČ)—using two distinct visual scoring approaches.

- Method 1: The entire tumour section was reviewed at 100× magnification, and the percentage of positively stained tumour-cell nuclei was estimated semi-quantitatively to the nearest 1%.
- Method 2: Only the invasive front was assessed at 200× magnification; five consecutive fields were examined, and exactly 500 tumour cells were counted (100 cells per field). The proportion of positive nuclei among these cells yielded the quantitative value.



a) b)

Figure 1. illustrates both techniques: (a) whole-section semi-quantitative scoring at 100×, and (b) focused quantitative scoring at the invasive margin at 200× (arrow).

Flow-cytometric determination of S-phase fraction

SPF was measured by flow cytometry as described previously [15]. Fine-needle aspiration biopsy (FNAB) samples from invasive cancers were processed according to the protocol introduced by Otto *et al*. [35] and later adapted for local use according to Pogačnik *et al*. [36]. Measurements were performed with a PAS II Flow Cytometer (Partec, Münster, Germany). For each sample, 5000–65, 000 events were acquired at a rate of approximately 200 counts per second, generating a 1024-channel DNA histogram.

Instrument calibration, performed before acquisition, employed trout erythrocytes and human lymphocytes to define diploid peaks and optimize the G0/G1 coefficient of variation (CV) to 1.5–2%.

- For diploid tumours, SPF represented the number of events located between the G0/G1 and G2/M peaks divided by the sum of all events in G0/G1, G2/M, and the S-phase window.
- For aneuploid tumours, the calculation followed the same logic but used only the aneuploid cell cycle distribution.

Data analysis

Continuous variables were summarised as medians with interquartile ranges (IQRs), while categorical variables were presented as counts and percentages. Box-plots were used to display the distributions of SPF and Ki-67. Because Ki-67 values were highly skewed, they were log-transformed [37]. Group comparisons were performed using Pearson's chi-square or Fisher's exact test for categorical data and unpaired Student's t-tests for continuous variables. A p-value < 0.05 indicated statistical significance; no corrections for multiple testing were applied.

Inter-observer reliability for Ki-67 scoring was evaluated using the intra-class correlation coefficient (ICC) [38]. ICC values and corresponding 95% confidence intervals were calculated in SPSS v.22 using a mean-rating model ($k = 3$), absolute-agreement definition, and two-way random-effects design. Reliability was categorized as <0.5 (poor), 0.5–0.75 (moderate), 0.75–0.90 (good), and >0.90 (excellent).

To visualize concordance and detect potential systematic discrepancies across methods, Bland–Altman plots were generated for the proliferation markers (SPF and Ki-67 Methods 1 and 2) [39].

Relationship between SPF and Ki-67

Associations between SPF and both Ki-67 assessment techniques were illustrated using scatterplots. Kendall's rank correlation was applied to quantify the relationship between the two IHC-based Ki-67 measurements and SPF, as this coefficient is more resistant to outliers than Spearman's. Calculations used pairwise-complete datasets, and 95% confidence intervals (CI) were generated through 104 bootstrap resamples. According to published guidelines, Kendall's tau values of 0.26–0.48 reflect a moderate association, while coefficients of 0.49–0.70 indicate a strong positive relationship [40].

Kaplan–Meier estimators were used to compute curves for time to recurrence (TTR), recurrence-free survival (RFS), and overall survival (OS). TTR was defined as the interval from surgery to relapse; RFS encompassed time to first recurrence or death; OS captured mortality from any cause. Individuals without an event were censored at their final follow-up. For prognostic analyses, the primary scoring strategy for Ki-67 (Method 1) was selected because it reflects routine clinical practice. Clinical interpretation of Ki-67 followed IKWG recommendations [27] (≤5% = low; ≥30% = high; 6–29% = intermediate), and additionally the ESMO and St. Gallen criteria [12, 13, 31] (≤10% = low; ≥30% = high; 11–29% = intermediate). For SPF, a cut-off of 6.7% was applied based on earlier findings [15]. All clinically meaningful pathological and clinical variables were incorporated into the survival models. Kaplan–Meier curves were compared using log-rank tests, and prognostic effects were tested with both Cox proportional hazards and time-dependent Cox models.
Statistical work was conducted using R for Windows v2022.07.01, Excel for Windows, and SPSS v.22.

Results and Discussion

Patients
A total of 690 individuals with invasive breast carcinoma treated between 1992 and 1997 at the Institute of Oncology Ljubljana were initially considered, of whom 411 satisfied all inclusion criteria **(Figure 2)**.

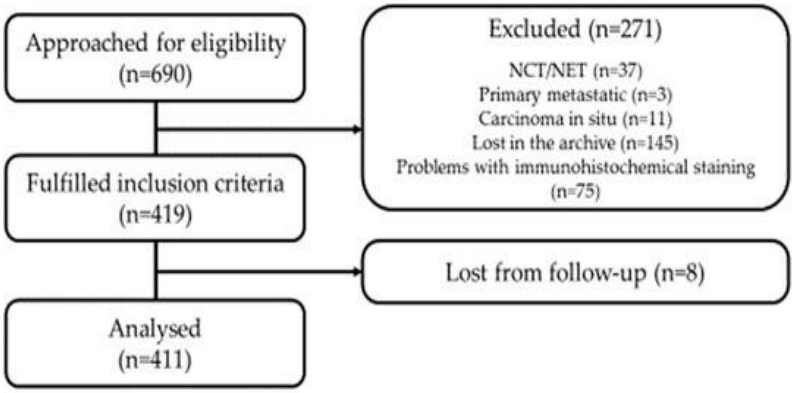


Figure 2. depicts the patient flow, including exclusions due to NCT (neoadjuvant chemotherapy) and NET (neoadjuvant endocrine therapy).

Patient and tumour characteristics are summarized in **Table 1**. All participants were women, with a median age of 58.9 years (IQR 46.7–67.2); 70.1% were aged ≥50 years. Most had invasive carcinoma of no special type (NST) (350; 85.2%), while 51 (12.4%) had invasive lobular carcinoma and 10 (2.4%) represented other uncommon histologies. Nearly half (48.4%) had grade 3 tumours, whereas only 10.2% had grade 1 disease by the Nottingham system. Tumour stage 2 was identified in 53.8%, axillary nodes were negative in 55.2%, and 68.7% of tumours were ER-positive. HER2 assessment was not performed at that time.

Table 1. lists baseline clinical and tumour data for the full cohort (left portion) and stratified by SPF and Ki-67 Method 1 results (right portion).

Characteristic	**n (%)**							
	All Patients n = 411 (100)	**Low SPF (<6.7%) 219 (47)**	**High SPF (≥6.7%) 218 (53)**	**p-Value**	**Low Ki-67 1–10% 122 (29.7)**	**Ki-67 11–29% 193 (47.0)**	**High Ki-67 ** ≥30% ** 96 (23.3)**	**p-Value**
Age **median (IQR)**	58.9 (46.7–67.2)	60.5 (47.7–68.6)	58.3 (46.7–65.4)	0.075	58.9 (47.7–68.3)	60.5 (49.2–67.2)	54.8 (44.4–65.4)	0.042 [1] 0.023 [2]
Age **n (%)**				0.704				0.087
<50 years	123 (29.9)	56 (45.5)	67 (54.5)		36 (29.3)	50 (40.6)	37 (30.1)	
≥50 years	288 (70.1)	137 (47.6)	151 (52.4)		86 (29.9)	143 (49.6)	59 (20.5)	

Histology **n (%)**				0.077			0.010
NST	350 (85.2)	158 (45.1)	192 (54.9)		94 (26.9)	170 (48.6)	86 (24.6)	
ILC and other	61 (14.8)	35 (57.4)	26 (42.6)		28 (45.9)	23 (37.7)	10 (16.4)	
Grade **n (%)**				<0.0001			<0.0001
Grade 1	42 (10.2)	33 (78.6)	9 (21.4)		31 (73.8)	11 (26.2)	0 (0)	
Grade 2	170 (41.4)	104 (61.2)	66 (38.8)		76 (44.7)	88 (51.8)	6 (3.5)	
Grade 3	199 (48.4)	56 (28.1)	143 (71.9)		15 (7.6)	94 (47.2)	90 (45.2)	
LVI **n (%)**				0.011			0.339
Absent	218 (68.3)	111 (50.9)	107 (49.1)		74 (33.9)	101 (46.3)	43 (19.7)	
Present	191 (31.7)	36 (36.6)	65 (64.4)		26 (25.7)	53 (52.5)	22 (21.8)	
Tumour stage **n (%)**				0.038			0.010
pT1 (≤20 mm)	154 (37.5)	85 (55.2)	69 (44.8)		55 (35.7)	73 (47.4)	26 (16.9)	
pT2 (>20≤50)	221 (53.8)	93 (22.1)	128 (57.9)		53 (24)	110 (49.8)	58 (26.2)	
pT3 (>50 mm)	35 (8.5)	15 (42.9)	20 (57.1)		13 (37.1)	10 (28.6)	12 (34.3)	
Missing	1 (0.2)							
Lymph nodes **n (%)**				0.260			0.559
pN0 (negative)	227 (55.2)	116 (51.1)	111 (48.9)		63 (27.7)	108 (47.6)	56 (24.7)	
pN1 (1–3 positive)	100 (24.3)	44 (44.0)	56 (56.0)		35 (35.0)	44 (44.0)	21 (21.0)	
pN2 (4–9 positive)	49 (11.9)	20 (40.8)	29 (59.2)		15 (39.6)	26 (53.1)	8 (16.3)	
pN3 (≥10 positive)	35 (8.5)	13 (37.1)	22 (62.9)		9 (25.7)	15 (42.9)	11 (31.4)	
ER **n (%)**				<0.0001			<0.0001
ER negative	126 (31.3)	38 (30.2)	88 (69.8)		24 (19.0)	44 (35.0)	58 (46.0)	
ER positive	276 (68.7)	151 (54.7)	125 (45.3)		96 (34.8)	145 (52.5)	35 (12.7)	

ER = estrogen receptor; IQR = interquartile range; LVI = lymphovascular invasion; NST = invasive carcinoma of no special type; SPF = S-phase fraction. Bold values indicate statistically significant associations.
1 = low vs. high Ki-67; 2 = intermediate vs. high Ki-67.

Ki-67 scoring results

Figure 3 summarizes the Ki-67 results obtained by three independent readers using both scoring strategies. Method 1 produced a significantly lower mean Ki-67 value than Method 2 (22% vs. 25.1%, p = 0.02). The median Ki-67 was 16.3% (Q1 9.3, Q3 28.3) using Method 1 and 18.7% (Q1 11.3, Q3 32.7) for Method 2. Ki-67 values showed non-normal distributions; therefore, log2 transformation was applied for parametric analyses.

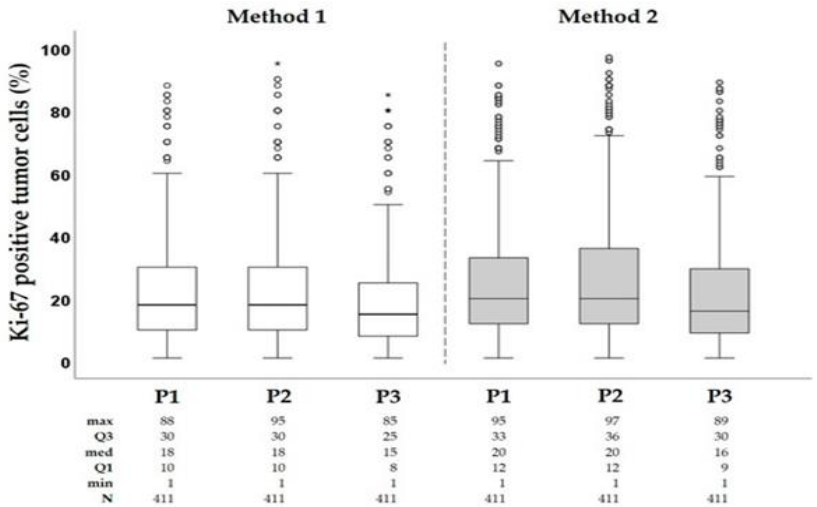
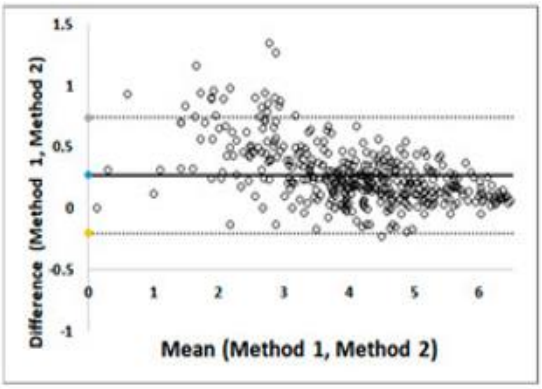


Figure 3. shows the box-plots, in which the box edges reflect Q1 and Q3, the median is marked by the interior line, and whiskers extend to values within 1.5×IQR. Outliers and extreme outliers appear as circles and stars. P1, P2, P3 correspond to the three evaluating pathologists.

Agreement and correlation for Ki-67 scoring

Intraclass correlation coefficients **(Table 2)** were calculated separately for each Ki-67 method across the three readers. For Method 1, reproducibility was excellent, with ICC values above 0.90 and the 95% CI remaining above this threshold. For Method 2, performance ranged from good to excellent, as ICC values exceeded 0.90, although the lower confidence limit fell below 0.90, indicating slightly more variability. Overall, interobserver consistency within the laboratory was very high.

Agreement between Method 1 and Method 2 was visualized using Bland–Altman plots **(Figure 4b)**. The mean difference was small and the agreement limits were tight; however, discrepancies were more pronounced at lower Ki-67 levels, indicating that the two techniques should not be used interchangeably at the low end of the range. Despite this, the correlation between the two methods was remarkably strong, with Kendall's tau = 0.92 (95% CI 0.91–0.93, $p < 0.0001$).



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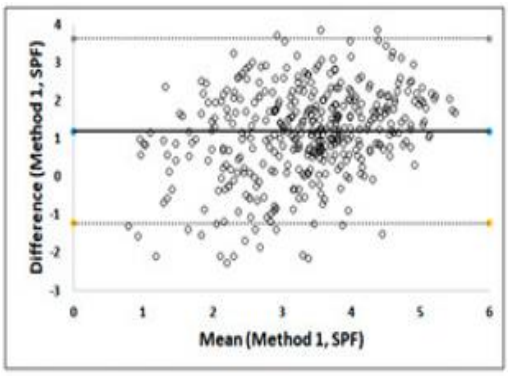

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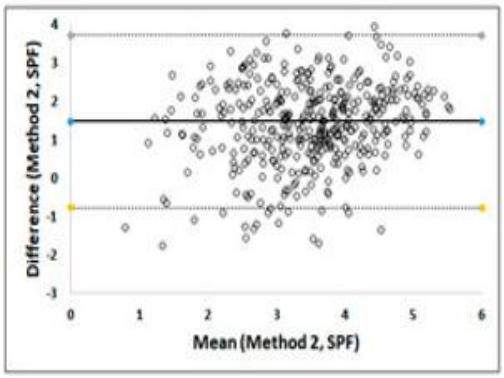


c)

Figure 4. displays Bland–Altman graphs comparing M1 vs. M2, M1 vs. SPF, and M2 vs. SPF (log2-transformed). The y-axis shows differences, while the x-axis shows means. The center line marks the mean difference; dashed lines indicate the upper and lower limits of agreement. For each Ki-67 method, the average of the three readers' assessments was used. Blue symbols represent median differences; yellow and grey symbols depict lower and upper limits.

Table 2. presents ICC estimates with 95% CIs based on mean-rating (k = 3), absolute-agreement, two-way random-effects modeling. Interpretation used single-measure outputs.

Intraclass Correlation	Single Measures	95% Confidence Interval		F-Test with True Value 0			
		Lower Bound	Upper Bound	Value	df1	df2	Sig.
Method 1	0.962	0.926	0.978	110.934	410	410	0.000
Method 2	0.946	0.888	0.969	79.217	410	820	0.000

Agreement and correlation between Ki-67 and SPF

Figure 5 illustrates the relationship between SPF and the Ki-67 measurements obtained with Methods 1 and 2 by the three evaluators (upper panel: Method 1; lower panel: Method 2). As anticipated, Ki-67 values for each

specimen exceeded SPF values, since the overall pool of cycling cells is larger than the subset in S phase. Bland–Altman plots using log2-converted data were applied to assess concordance. These demonstrated minimal systematic deviation and generally solid agreement between SPF and both Ki-67 techniques **(Figures 4a and 4c)**. Still, the association between them reached only moderate strength: Kendall's τ was 0.33 (95% CI 0.26–0.38; $p < 0.0001$) for Method 1 and 0.33 (95% CI 0.26–0.39; $p < 0.0001$) for Method 2.

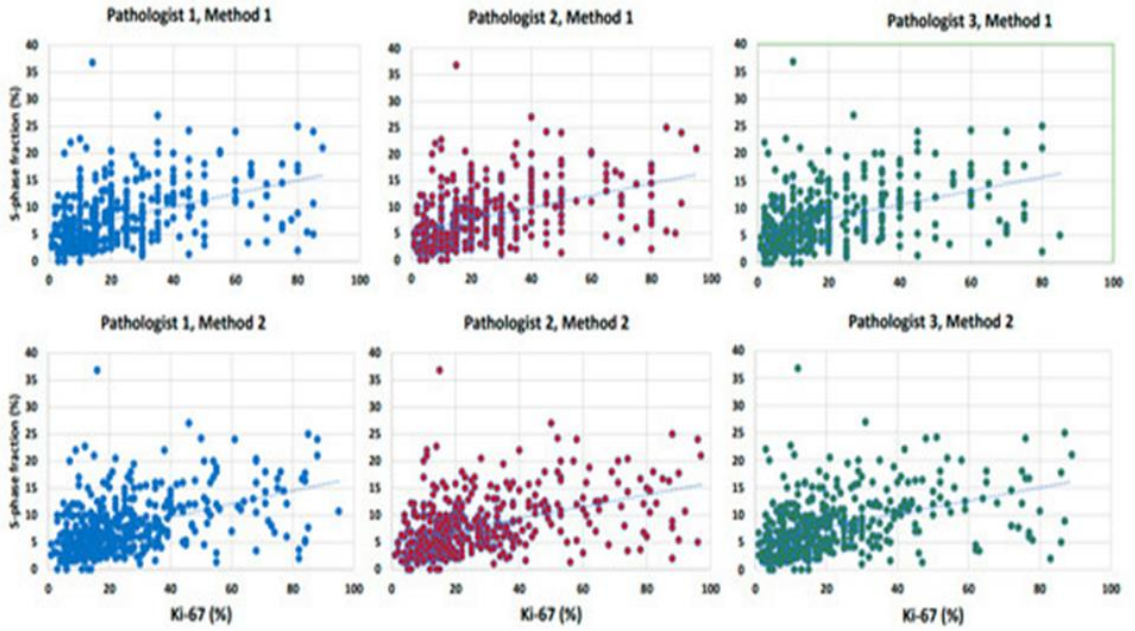


Figure 5. Scatter diagrams comparing each Ki-67 scoring approach with the S-phase fraction. M1 = Method 1; M2 = Method 2 (continuous outputs).

Association of clinic-pathological characteristics with proliferative indexes SPF and Ki-67

For prognostic evaluation related to clinicopathologic variables, only Method 1 Ki-67 results were incorporated, given that this approach is the standard in routine diagnostics at the Institute of Oncology Ljubljana.

High-SPF tumors (≥6.7%) appeared significantly more often as grade 3, ER-negative, ≥pT2 stage, and with lymphatic involvement. Likewise, tumors falling into the high Ki-67 group (≥30%) were enriched for grade 3, ER-negative, and ≥pT2 disease, and occurred more frequently in younger individuals compared with those in the low or intermediate categories **(Table 1)**. Notably, neither SPF nor Ki-67 distributions correlated with nodal status. Because inter-observer inconsistency in Ki-67 scoring remains a major challenge, international groups endorse distinct thresholds. Clinically, the intermediate Ki-67 range is the most problematic for therapeutic decision-making. Using the ESMO definition (11–29%), this subgroup comprised 47% of the cohort **(Table 1)**. Under the IKWG criteria (6–29%), the intermediate population increased to 62.8%. Accordingly, our analyses emphasized comparisons of this middle category with the low- and high-proliferation groups.

Treatment and outcome

Table 3 summarize management strategies and follow-up outcomes. Mastectomy was performed in 66.7% of cases. Axillary dissection—standard at the time—was completed for every patient in this retrospective dataset. Adjuvant radiotherapy was administered to 36% of the cohort. Chemotherapy was given to 50.4% (predominantly CMF; 13.8% received anthracycline regimens), while endocrine therapy (tamoxifen and/or goserelin) was delivered in 56%. Individuals with elevated SPF or Ki-67 markers were notably more likely to undergo chemotherapy and less likely to receive endocrine therapy; the high-SPF group further showed greater use of radiotherapy. Marked differences in systemic therapy were observed between pre- and post-menopausal patients ($p < 0.001$): ET, CT, and combined CT-ET were used in 8.6%, 46.4%, and 29% of pre-menopausal patients versus 51.2%, 12.6%, and 20.3% in post-menopausal patients, respectively. At that time, endocrine treatment was routinely favored for post-menopausal women but not for younger patients.

Table 3. Key treatment approaches and outcomes relative to Ki-67 and SPF status.

Type of Treatment	All Patients (n = 411)	Low SPF (<6.7%) (n = 193)	High SPF (≥6.7%) (n = 218)	p-Value	Low Ki-67 1–10% 122 (29.7)	Intermediate Ki-67 11–29% 193 (47.0)	High Ki-67 ≥30% 96 (23.3)	p-Value
Type of surgery				0.780				0.864
Mastectomy	274 (66.7)	130 (47.4)	144 (52.6)		83 (30.3)	129 (47.1)	62 (22.6)	
BCS	137 (33.3)	63 (46)	74 (54)		39 (28.5)	64 (46.7)	34 (24.8)	
Adjuvant RT	148 (36)	57 (38.5)	91 (61.5)	0.010	42 (28.4)	65 (43.9)	41 (27.7)	0.293
Adjuvant ET	224 (55.7)	116 (51.8)	108 (42.8)	0.001	69 (30.8)	115 (51.3)	40 (17.9)	<0.0001
ER negative	50 (22.3)	16 (32)	34 (68)		9 (18)	20 (40)	21 (42)	
ER positive	174 (77.7)	100 (57.5)	74 (42.5)		60 (34.5)	95 (54.6)	19 (10.9)	
Adjuvant CMF	207 (50.4)	86 (41.5)	121 (58.5)	0.001	54 (26.5)	92 (45.1)	58 (28.4)	<0.0001
ER negative	84 (41.2)	23 (27.4)	62 (72.6)		15 (17.9)	26 (31)	43 (51.1)	
ER positive	120 (58.8)	62 (51.7)	58 (48.3)		39 (32.5)	66 (55)	15 (12.5)	
Events								
Relapse	221 (53.1)	99 (44.8)	122 (55.2)	0.213	56 (25.3)	112 (50.2)	53 (24.0)	0.104
Death	316 (76.9)	151 (47.8)	165 (52.2)	0.540	92 (29.1)	155 (49.1)	69 (21.8)	0.249

(Abbreviations: CMF = cyclophosphamide/5-fluorouracil/methotrexate; BCS = breast-conserving surgery; ET = endocrine therapy; RT = radiation therapy. Bold values indicate statistical significance.)

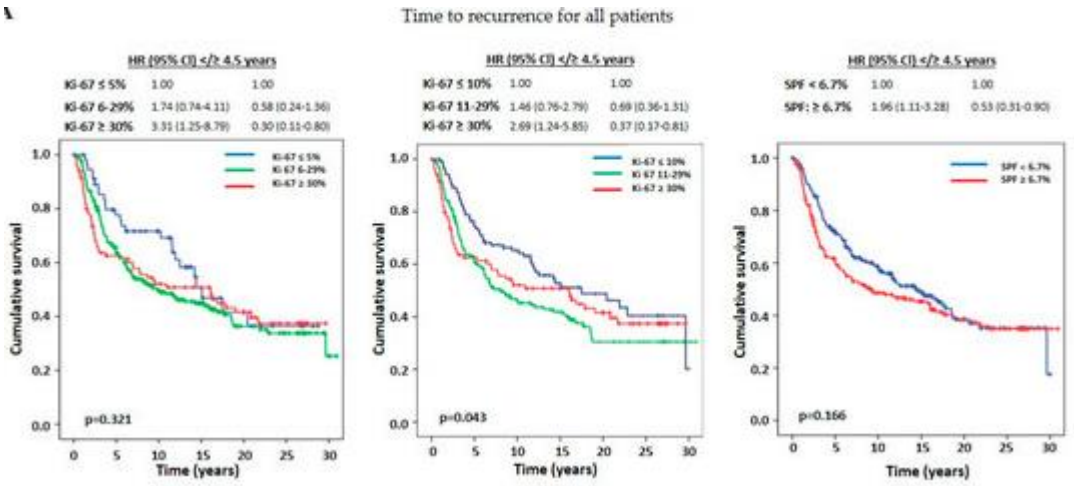
The median observation period was 26.8 years (range 0.2–30.9). Across the 411 individuals, 221 (53.1%) experienced recurrence, and 316 (76.9%) died **(Table 3)**. While the total relapse and mortality counts showed no major difference across SPF and Ki-67 strata during this extended follow-up, patterns of recurrence varied (details in Section 3.5).

Survival analysis

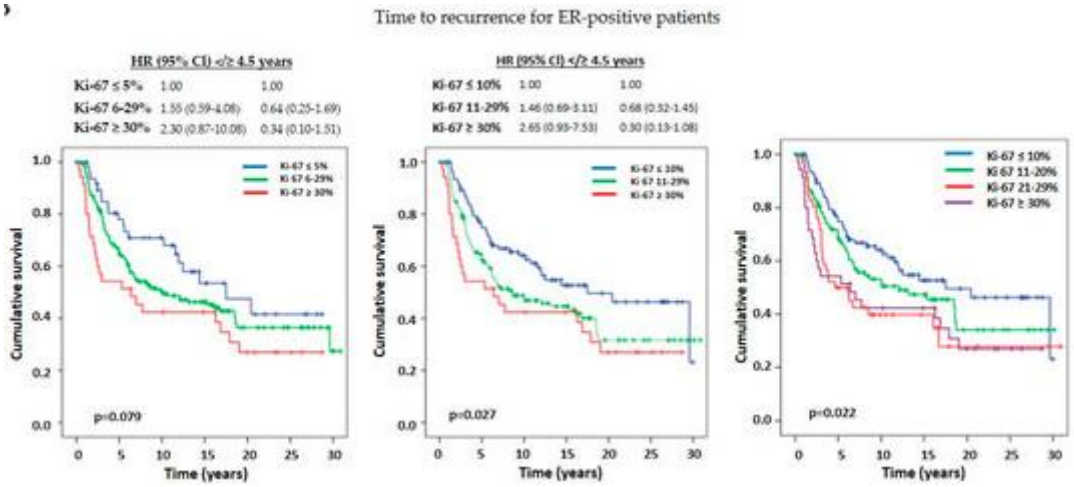
Briefly, median RFS was 8.6 years (95% CI 6.4–10.8), and median OS was 12.3 years (95% CI 9.9–14.6). With long-term monitoring, as in this dataset, deaths predominate after the 10-year mark, meaning TTR may offer a more accurate representation of recurrence dynamics than RFS.

Time to recurrence according to SPF and Ki-67 in all patients

Time-varying hazard ratios for SPF and Ki-67 are shown in **Figure 6a**. During the initial 4.5 years post-surgery, recurrence risk was markedly higher in the high-proliferation groups: HR 3.31 (p = 0.016) for Ki-67 ≥ 30% vs. ≤5%; HR 2.69 (p = 0.012) for Ki-67 ≥ 30% vs. ≤10%; and HR 1.96 (p = 0.020) for SPF ≥ 6.7% vs. <6.7%. Beyond 4.5 years, the low-proliferation categories displayed higher recurrence hazards than the high groups. The intermediate-risk class (6–29%) had an early hazard positioned between the high and low categories, but later exceeded both, which corresponds to the crossover appearing around year 5 in the survival curves. This may reflect higher early mortality in the high-risk groups, reducing recurrence as a competing event. Interestingly, using the alternative intermediate Ki-67 cut-off (11–29%) yields an early hazard profile closely resembling that of the high-Ki-67 group.



a)



b)

Figure 6. Kaplan–Meier plots for recurrence among (a) the full cohort and (b) ER-positive patients, stratified into three Ki-67 groups (with alternate cut-offs) and two SPF groups. High Ki-67 and high SPF predict increased early relapse, while low Ki-67 and low SPF correspond to later recurrences. For all patients, a 5% Ki-67 threshold more clearly separates low vs. high risk, whereas 10% performs better in ER-positive cases.

Time to recurrence according to Ki-67 groups in ER-positive patients

We concentrated our analysis primarily on the ER-positive population (n = 276; 68.7%), paying particular attention to the intermediate Ki-67 category. **Figure 6b** displays the TTR patterns for the different intermediate cut-offs. In our material, the three Ki-67 strata remained distinctly separated for the entire observation period, with no intersections of the curves. Notably, the rightmost panel shows that the trajectory of the 21–29% subgroup closely mirrors that of the Ki-67 ≥ 30% cohort, implying that a threshold around ≥20% might mark the onset of a high-risk Ki-67 profile. These findings, however, reflect only this dataset and require validation in future studies. We also conducted an exploratory TTR evaluation based on systemic therapy in ER-positive patients with pN0–1 disease (n = 226) **(Figure 7)**. Within the low-risk Ki-67 group (≤10%), individuals managed solely with ET demonstrated lower hazard ratios than those treated with CT or combined CT-ET (p = 0.033). Among intermediate cases (11–29%), ET and CT-ET yielded comparable TTR outcomes (p = 0.154). These observations raise the possibility that intermediate Ki-67 tumors might not benefit from chemotherapy, while in the low Ki-67 category, CT may even have been harmful—although further confirmation is essential.

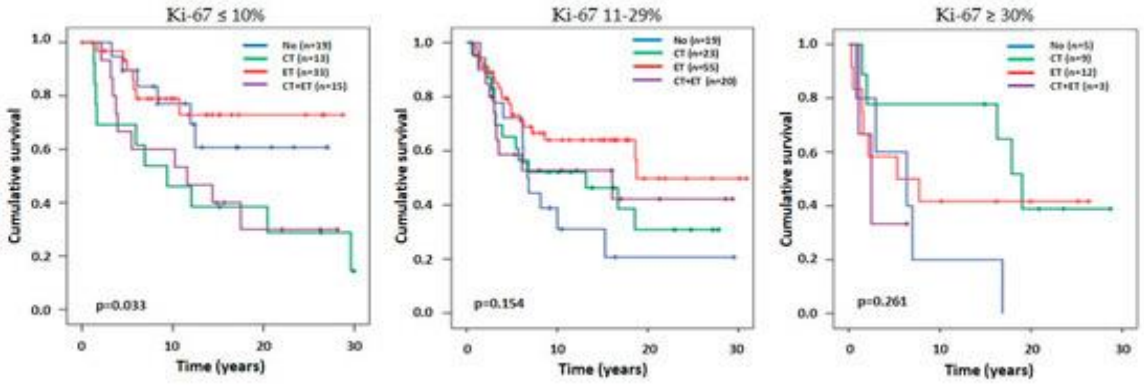


Figure 7. Kaplan–Meier curves illustrating TTR by treatment type in ER-positive patients with 0–3 involved lymph nodes (pN1) across the three Ki-67-defined risk groups.
ET = endocrine therapy; CT = chemotherapy.

Univariate and multivariate survival analysis

We assessed the prognostic importance of SPF, Ki-67, and other clinicopathologic parameters. **Table 4** summarizes the results for TTR, RFS, and OS. In multivariate Cox modeling, only nodal status and lymphovascular invasion emerged as independent predictors for TTR. Tumors classified as pN2 and pN3 carried 2.2- and 3.75-fold higher hazards of progression relative to pN0 disease, respectively. Likewise, lymphovascular invasion was associated with a 1.98-fold increase in hazard compared with its absence. Ki-67 did not satisfy the proportional hazards assumption for recurrence (**Figures 6a and 6b**).

For RFS and OS, independent prognostic indicators included age ≥ 50 years, lymphovascular invasion, and nodal involvement. Patients aged ≥50 showed 1.83- and 2.29-fold higher risks for events (RFS and OS) over the 28-year follow-up compared with those under 50. Lymphovascular invasion conferred hazard ratios of 1.51 (RFS) and 1.41 (OS). Individuals with ≥4 positive nodes experienced a 2–3-fold elevation in the likelihood of recurrence or death, whereas those with 1–3 positive nodes did not differ significantly from node-negative patients.

Table 4. Univariable and multivariable analyses of predictors for TTR, RFS, and OS. (NST = invasive carcinoma of no special type; ILC = invasive lobular carcinoma; ER = estrogen receptors; LVI = lymphovascular invasion; SPF = S-phase fraction.)

	Time to Progression				**Relapse-Free Survival**				**Overall Survival**			
	Univariable HR (95%CI)	**p-Value**	**Multivariable HR (95% CI)**	**p-Value**	**Univariable HR (95% CI)**	**p-Value**	**Multivariable HR (95% CI)**	**p-Value**	**Univariable HR (95% CI)**	**p-Value**	**Multivariable HR (95% CI)**	**p-Value**
Age < 50 years	1.00	0.214	/	/	1.00	<0.0001	1.00	<0.0001	1.00	<0.0001	1.00	<0.0001
Age ≥ 50 years	1.20 (0.90–1.61)				1.80 (1.40–2.32)		1.83 (1.36–2.47)		2.25 (1.72–2.94)		2.29 (1.66–3.15)	
Histology												
NST	1.00	0.506	/	/	1.00	0.753	/	/	1.00	0.592	/	/
ILC + other	1.13 (0.79–1.60)				1.05 (0.78–1.41)				0.92 (0.67–1.25)			
Grade												
Grade 1	1.00	0.082	1.00	0.518	1.00	0.206	/	/	1.00	0.077	1.00	0.397
Grade 2	1.50 (0.89–2.52)	0.126	1.23 (0.63–2.41)	0.539	1.41 (0.96–2.06)	0.077			1.55 (1.04–2.30)	0.032	1.27 (0.78 (2.09)	0.340

Grade 3	1.75 (1.05–2.91)	0.031	1.41 (0.72–2.74)	0.316	1.29 (0.89–1.88)	0.180			1.55 (1.04–2.29)	0.030	1.40 (0.85–2.30)	0.185
Tumor stage												
pT1 (≤20 mm)	1.00	<0.0001	1.00	0.898	1.00	<0.0001	1.00	0.489	1.00	0.001	1.00	0.772
pT2 (21–50 mm)	1.49 (1.11–1.99)	0.008	1.04 (0.74–1.48)	0.809	1.42 (1.13–1.80)	0.003	1.07 (0.81–1.41)	0.635	1.37 (1.08–1.74)	0.010	1.00 (0.75–1.33)	0.994
pT3 (>50 mm)	2.73 (1.74–4.28)	<0.0001	0.92 (0.50–1.70)	0.797	2.60 (1.75–3.82)	<0.0001	1.39 (0.81–2.38)	0.232	1.98 (1.33–2.95)	0.001	1.20 (0.71–2.02)	0.505
Nodal stage												
pN0 (negative)	1.00	<0.001	1.00	<0.0001	1.00	<0.0001	1.00	<0.0001	1.00	<0.0001	1.00	<0.0001
pN1 (1–3 positive)	1.19 (0.86–1.65)	0.294	1.23 (0.83–1.82)	0.310	1.05 (0.80–1.36)	0.747	1.81 (0.86–1.62)	0.301	1.04 (0.79–1.37)	0.792	1.31 (0.95–1.81)	0.102
pN2 (4–9 positive)	2.29 (1.56–3.37)	<0.0001	2.20 (1.40–3.46)	0.001	2.33 (1.68–3.24)	<0.0001	2.13 (1.47–3.09)	<0.0001	2.69 (1.92–3.77)	<0.0001	2.72 (1.85–4.00)	<0.0001
pN3 (≥10 positive)	3.98 (2.61–6.08)	<0.0001	3.75 (2.20–6.37)	<0.0001	43.21 (2.20–4.68)	<0.0001	2.52 (1.57 (4.07)	<0.0001	3.35 (2.92–4.89)	<0.0001	2.84 1.79–4.53)	<0.0001
LVI												
No	1.00	<0.0001	1.00	<0.0001	1.00	<0.0001	1.00	0.003	1.00	0.007	1.00	0.022
Yes	2.37 (1.74–3.23)		1.98 (1.42–2.76)		1.70 (1.31–2.21)		1.54 (1.16–2.04)		1.45 (1.1–1.89)		1.40 (1.05–1.88)	
ER negative	1.00	0.917	/	/	1.00	0.571	/	/	1.00	0.729	/	/
ER positive	0.99 (0.74–1.31)				1.07 (0.84–1.36)				1.04 (0.82–1.33)			
Low SPF (<6.7%)	1.00	0.232	/	/	1.00	0.530	/	/	1.00	0.409	/	/
High SPF (≥6.7%)	1.18 (0.90–1.53)				1.07 (0.86–1.33)				1.10 (0.88–1.37)			
Ki-67												
Low (≤5%)	1.00	0.324	/	/	1.00	0.623	/	/	1.00	0.760	/	/
Intermediate (6–29%)	1.38 (0.90–2.12)	0.136			1.08 (0.78–1.49)	0.649			1.05 (0.76–1.46)	0.760		
High (≥30%)	1.35 (0.84–2.18)	0.214			0.95 (0.65–1.38)	0.784			0.95 (0.65–1.39)	0.794		

In this retrospective cohort of patients with early-stage breast carcinoma, we examined the concordance and correlation between two Ki-67 scoring strategies based on IHC. First, we observed extremely high agreement among the three pathologists for both the whole-slide evaluation (Method 1) and the invasive-front-focused approach (Method 2). Second, each Ki-67 approach showed good alignment with SPF—a quantitative flow-cytometric metric—although the correlation between Ki-67 and SPF was only moderate, while the correlation between the two Ki-67 methods themselves was strong. For downstream prognostic evaluation, the visual method was selected.

Both proliferation markers (Ki-67 and SPF) displayed time-dependent effects: the high-proliferation groups (Ki-67 ≥ 30% and SPF ≥ 6.7%) exhibited an elevated early recurrence risk, which reversed after 4.5 years, at which point the low-proliferation categories showed greater risk. Among ER-positive patients, the intermediate Ki-67 category—regardless of cut-off—remained consistently separated from both low and high groups across time.

However, the intermediate curve aligned more closely with the high Ki-67 line (≥30%) than with the low group (≤5% or ≤10%). In ER-positive pN0–1 patients, ET alone produced outcomes comparable to CT or CT-ET in the intermediate Ki-67 subset. Within our dataset, a Ki-67 value around 20% appeared to best discriminate low- from high-proliferation tumors.

Although Ki-67 remains an important biomarker for prognosis and prediction in breast cancer, routine clinical use is hampered by variable reproducibility due to nonstandardized protocols and inter-observer differences. Despite recommendations from the IKWG [27, 41], multiple assessment strategies are still in practice [42-45]. In the present study, the routinely applied Method 1 demonstrated exceptionally high concordance (ICC > 0.90), suggesting robust reproducibility within our laboratory. This is somewhat unexpected given the historically reported limitations of visual Ki-67 scoring [46, 47]. The strong agreement may reflect the long-standing collaboration between the two breast pathology specialists in our group, though even the pathology resident—despite minimal experience—achieved excellent concordance, indicating that the method is straightforward to learn. Comparable findings have been reported by Hida *et al*. who showed that rapid visual grading using 5- and 10-point scales can effectively classify luminal breast cancers [48, 49]. A recent study also found good reproducibility for visual IKWG-guided Ki-67 scoring on core biopsies [50]. However, IKWG-based scoring requires about 9–13 minutes per case, whereas Method 1 is notably faster. Similar ICC results were also documented by del Rosario *et al*. [45]. Although "eyeballing" is inherently subjective, it remains practical, and some authors argue that evaluating the entire slide may even yield a more accurate reflection of proliferative activity [51].

We also found a strong association between the visual method and the quantitative approach that counted proliferating cells at the tumor's invasive edge (Method 2). However, the Bland-Altman analysis indicated that these two Ki-67 evaluation methods are not interchangeable. Although both approaches yielded comparable Ki-67 ranges, the average and median Ki-67 levels were higher with Method 2. This aligns with previous studies showing that methods focusing on the tumor's invasive margin tend to report elevated Ki-67 values compared to whole-slide analysis [52]. Additionally, we observed a solid concordance between each Ki-67 technique and SPF—a quantitative metric of cell proliferation with established prognostic value in our cohort [15] and in other studies [18, 19-23, 53]. The modest correlation in cases with higher Ki-67 may stem from the fact that Ki-67 is expressed in all cell cycle stages except G0, whereas SPF only measures cells in S phase. Furthermore, SPF might also reflect proliferation in non-cancerous cells within the cytological sample, such as stromal cells and infiltrating lymphocytes [4].

Recently, automated digital image analysis and AI-assisted methods for measuring proliferation in breast cancer have been suggested, improving consistency between observers [43, 45, 50, 54-58]. While digital approaches may eventually replace manual counting, their adoption is limited by cost and time—most require pathologists to mark regions for analysis [43]. Visual evaluation tends to underestimate Ki-67 activity [44, 54], so threshold adjustments are needed when new methods are implemented. For Method 1, mean and median Ki-67 values were 22% and 16.3%, respectively, consistent with other reports [45, 54].

There is still no agreement on the optimal Ki-67 cut-off. The marker's subjective nature and tumor heterogeneity contribute to high observer variability (around 5–30%). As early as 2001, Spyratos *et al*. highlighted this issue [53], recommending a 10% cut-off to exclude slow-proliferating tumors from chemotherapy and a 25% threshold to identify chemo-sensitive tumors. A meta-analysis involving 46 studies and 12, 155 patients reported Ki-67 cut-offs from 3.5% to 34%, determined by diverse criteria like mean or median values. High Ki-67 was associated with poorer DFS (HR 1.93) and OS (HR 1.95) [14], though its independent prognostic value remains unclear [59]. In this study, we evaluated outcomes using internationally endorsed Ki-67 cut-offs [27, 31]. Both guidelines classify Ki-67 ≥ 30% as high proliferation. In our cohort, 23.3% of patients met this high threshold. Using IKWG's low cut-off (≤5%) and ESMO's (≤10%), 13.9% and 29.7% of patients, respectively, were categorized as low Ki-67. Most tumors fell into the intermediate category (62.8% using IKWG and 47.0% using ESMO), creating a prognostic grey zone. We used ESMO criteria for further analysis to reduce the uncertain group's size.

Assessment of clinical and pathological variables revealed that patients with Ki-67 ≥ 30% and SPF ≥ 6.7% were more likely to present with grade 3, ER-negative, and ≥pT2 tumors compared to those in lower categories. Elevated Ki-67 and SPF correlated with reduced TTR in the first 4.5 years **(Figure 6a)**, with recurrence risk 2–3 times higher than in lower Ki-67 or SPF groups. Interestingly, beyond this period, recurrence risk doubled in low Ki-67 or SPF groups relative to the high groups. A Ki-67 cut-off of 5% was more effective in distinguishing between low and high-risk patients, regardless of ER status. The intermediate Ki-67 group remained ambiguous

in terms of prognosis and treatment decisions, especially in ER-positive cases. Kaplan-Meier analysis showed that the intermediate group's TTR tracked closely with the high-risk group **(Figure 6b)**. For ER-positive patients, an ESMO cut-off (Ki-67 ≤ 10%) was more useful for prognosis and chemotherapy decisions. However, in this population, a threshold of 20% might be optimal, as TTR curves for Ki-67 20–29% and ≥30% overlapped **(Figure 6b)**.

Currently, Ki-67 assessment is ideally supplemented with genomic assays like Oncotype DX to better predict recurrence and chemotherapy benefit. For example, in the PlanB trial (West German Study Group), chemotherapy was avoided in patients with Oncotype DX recurrence scores (RS) between 0 and 11 [60]. The ADAPT study demonstrated that in HR+/HER2− cancers with RS 12–25, patients who achieved Ki-67 ≤ 10% after 2–4 weeks of endocrine therapy could safely omit chemotherapy [61]. In the POETIC trial, post-menopausal women with low baseline Ki-67 had excellent 5-year outcomes without chemotherapy unless other poor prognostic factors were present [11]. In our cohort, ER-positive patients with Ki-67 ≤ 10% who received endocrine therapy alone had the best outcomes, while those who received chemotherapy or combined regimens did worse. However, given the small sample size for treatment evaluation, these survival curves should be viewed as exploratory and require confirmation in larger cohorts.

The performance of the intermediate Ki-67 category yielded notable outcomes **(Figure 6)**. There were no significant differences in recurrence rates between those treated with ET, CT, or CT-ET. However, the TTR curve suggested that patients receiving ET alone might have had the most favorable results. A significant observation from our data is that only 37.6% of premenopausal ER+ patients were given adjuvant ET or CT-ET. Meanwhile, 46.4% received CT, most commonly the CMF regimen, which poses a considerable risk of enduring ovarian suppression and thus lowers recurrence risk. This aligns with findings by Gray *et al*. [62], where ovarian suppression or ablation in ER-positive premenopausal women led to a 12.1% reduction in 15-year recurrence risk, along with reductions in breast cancer mortality (8%) and overall mortality (7.2%). Long-term benefits of ovarian suppression were also confirmed in extended results from the SOFT and TEXT trials [63].

Our multivariate analysis reinforced existing knowledge: nodal involvement and lymphovascular invasion remained key prognostic factors for recurrence and survival. Due to the long follow-up, age over 50 emerged as a factor influencing RFS and OS. However, Ki-67 could not be included in the proportional hazards model because of its changing impact over time, particularly in ER+ cases. Overall, high Ki-67 and SPF levels indicated greater early recurrence risk, while low levels predicted more late recurrences.

Strengths of this study include a large sample size and long-term follow-up. We adhered closely to pre-analytical and analytical standards for Ki-67 assessment. Our pathology unit undergoes frequent quality audits by UQ NEKAS and NORDIC and has a proven record. Only excision specimens were analyzed, with IHC conducted on full tissue sections. Rapid fixation (<72 hours post-excision) and sectioning just prior to staining minimized pre-analytical variability. Pathologists reached a consensus on each Ki-67 method in advance. Limiting assessments to a single cancer center ensured uniform processing.

Limitations include the retrospective nature and the exclusion of external pathologists and staining centers, which might have introduced greater variability due to different staining methodologies. Consistent with St. Gallen recommendations, we based Ki-67 interpretation on in-house median values [64]. Another concern is potential antigen decay from long-term paraffin block storage, which could reduce Ki-67 expression [65], though our observed values were on the higher end compared to similar studies [14]. Both IHC assessments aligned well with the quantitative flow cytometric SPF method. Lack of HER2 data is another limitation, as it was not routinely reported at the time, despite HER2-positive status being strongly linked to early recurrence. However, we focused mainly on luminal (ER-positive) cancers, with HER2+ cases accounting for only about 7% of the cohort.

Going forward, research will focus on advanced Ki-67 standardization techniques, such as cell line microarrays [66], and using artificial intelligence for image analysis. Improving quantitative proteomic methods for biomarker evaluation, like Ki-67, also shows promise [67], though challenges related to cost, availability, and validation may limit immediate clinical application.

Conclusion

Our study found good to excellent agreement between two Ki-67 scoring systems: a semi-quantitative whole-slide visual method and a quantitative invasive front method. Ki-67 was confirmed as a valuable prognostic and predictive biomarker. However, using traditional cut-offs to categorize patients into low and high Ki-67 groups

often leaves a large proportion—up to 50% (ESMO) or over 60% (IKWG)—in the intermediate category, which complicates prognosis and treatment selection.

In ER-positive breast cancer patients with low Ki-67, endocrine therapy (ET) appears most appropriate. For intermediate Ki-67 levels, ET typically performs similarly to CT-ET. Recently, gene expression tools like Oncotype DX are aiding decisions between ET and CT-ET. High Ki-67 indicates a greater risk of early recurrence and predicts chemotherapy sensitivity.

In conclusion, Ki-67 is an affordable and practical biomarker. When standardized in a central pathology setting, it proves useful in risk assessment and guiding treatment, especially in ER-positive breast cancers.

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