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Predictive Biomarkers and Personalized Medicine

Clinical Cancer Research

# Ki67 Measured after Neoadjuvant Chemotherapy for Primary Breast Cancer

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## Abstract

**Purpose:** The value of Ki67 measured on residual disease after neoadjuvant chemotherapy is not sufficiently described.

**Experimental Design:** Participants of the GeparTrio study with primary breast cancer randomly received neoadjuvant response-guided [8 cycles TAC (docetaxel/doxorubicin/cyclophosphamide) in responding and TAC-NX (vinorelbine/capecitabine) in nonresponding patients] or conventional (6 cycles TAC) chemotherapy according to interim response assessment. Ki-67 levels were centrally measured immunohistochemically after neoadjuvant treatment if tumor tissue was available. Here, we analyze 1,151 patients having a pathologic complete response (pCR; *n*, 484), or residual disease with low (0–15%), intermediate (15.1–35%), or high (35.1–100%) posttreatment Ki67 levels in 488, 77, and 102 patients, respectively.

**Results:** Patients with high posttreatment Ki67 levels showed higher risk for disease relapse ($P < 0.0001$) and death ($P < 0.0001$) compared with patients with low or intermediate Ki67 levels. Patients with low Ki67 levels showed a comparable outcome to patients with a pCR ($P = 0.211$ for disease-free and $P = 0.779$ for overall survival). Posttreatment Ki67 levels provided more prognostic information than pretreatment Ki67 levels or changes of Ki67 from pre- to posttreatment. Information on pCR plus posttreatment Ki67 levels surmount the prognostic information of pCR alone in hormone–receptor-positive disease [hazard ratios (HR), 1.82–5.88] but not in hormone–receptor-negative disease (HR: 0.61–1.73). Patients with conventional and response-guided treatment did not show a different distribution of posttreatment Ki67 ($P = 0.965$).

**Conclusions:** Posttreatment Ki67 levels provide prognostic information for patients with hormone–receptor-positive breast cancer and residual disease after neoadjuvant chemotherapy. Levels were not prognostic for outcome after response-guided chemotherapy. High posttreatment Ki67 indicates the need for innovative postneoadjuvant treatments. *Clin Cancer Res; 19(16); 4521–31. ©2013 AACR.*

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## Introduction

Neoadjuvant chemotherapy is a standard of care for patients with inoperable or high-risk operable breast cancer with the aim to reduce the extent of surgery (1). In addition, information on response obtained at surgery is used not only to assess long-term prognosis of patients but also as a short-term endpoint to evaluate efficacy of established treatments in an individual patient or of innovative regimens within a clinical trial situation. Pathologic complete response (pCR) is considered a surrogate efficacy endpoint generally correlated with favorable long-term outcome. However, a recent meta-analysis proposed that pCR is linked with a better prognosis for patients with hormone–receptor-negative (triple-negative or HER2-positive) tumors, but only for a minority of patients with hormone–receptor-positive tumors (2).

Another benefit of neoadjuvant chemotherapy was recently reported for the GeparTrio study: an interim-response–guided chemotherapy modification resulted in

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## Translational Relevance

This study shows the prognostic relevance of Ki67 in early breast cancer treated with neoadjuvant chemotherapy. Ki67 measured in chemoresistent residual tumor at the time of surgery provides more relevant prognostic information than measurement in pretreatment core biopsies. As having no pathologic complete response is not per se correlated with poor outcome in patients with residual hormone–receptor-positive tumors, low Ki67 levels can identify patients with a prognosis as good as patients with a pathologic complete response. Ki67 measured in residual hormone–receptor-negative tumors can further subdivide this unfavorable group of patients without a pathologic complete response. High Ki67 levels in residual disease predict a considerable risk of relapse and identify candidates for new antiproliferative treatment approaches (e.g., cyclin-D kinase inhibitors). Taken together, these data support retesting of Ki67 in patients with residual disease after neoadjuvant chemotherapy for better planning of subsequent therapies and intensity of surveillance.

a better overall survival when compared with conventional fixed-schedule chemotherapy (3). Participants of the GeparTrio study received two initial cycles of neoadjuvant chemotherapy, and response was clinically assessed thereafter. Patients with an interim response were randomized to four or six further cycles of the same chemotherapy, whereas patients without interim response were randomized to continue the same chemotherapy or to switch to another. No differences in pCR and sonographic response rates were observed between the randomized arms, respectively (4, 5). However, follow up revealed that patients being treated with the response-guided treatments showed a better disease-free and overall survival (OS) than patients being treated conventionally. This survival gain was observed only in patients with hormone–receptor-positive tumors (3).

Our hypothesis for this study was built on the earlier observation: for patients with hormone–receptor-positive tumors, pCR is an infrequent event not strongly correlated with prognosis. Therefore, pCR cannot forecast a survival benefit. Other surrogate efficacy endpoints after or during neoadjuvant chemotherapy for patients with hormone–receptor-positive tumors are, therefore, needed (6).

Tumor proliferation is considered to have the potential for such a new surrogate marker (7). If Ki67, a nuclear protein in cycling cells indicating tumor proliferation (8–10), is detected in a substantial fraction of tumor cells, patients are at high risk for relapse and death due to breast cancer (11, 12). High percentage of cells expressing Ki67 in the initial tumor biopsy was correlated with a higher rate of pCR after neoadjuvant chemotherapy (13–16), and preliminary studies suggest that Ki67 levels measured after neoadjuvant chemotherapy are of prognostic value for survival (17–20).

## Materials and Methods

### Objectives

The aim of this prospectively planned translational research project was to evaluate the prognostic and predictive impact of posttreatment Ki67 levels in surgical specimens from participants of the GeparTrio trial. We postulated that patients without a pCR to neoadjuvant chemotherapy are heterogeneous and can be subdivided by posttreatment Ki67 levels into subgroups with good or worse prognosis. We hypothesized that response-guided treatment increases the frequency of tumors with low posttreatment Ki67 levels compared with conventional treatment if they were hormone-receptor positive, but not if they were hormone-receptor negative.

### Patients

Patients with untreated unilateral or bilateral primary breast cancer were enrolled between September 2002 and August 2005 in the GeparTrio study after giving written informed consent. Eligibility required histologic confirmation of the diagnosis by core biopsy, plus at least one of the following risk factors: age less than 36 years, clinical tumor size more than5 cm, negativity of estrogen (ER) and progesterone (PR) receptor, clinical axillary node involvement, or undifferentiated tumor grade (G3). Full eligibility criteria have been provided elsewhere (5, 21, 22). All patients started treatment with two cycles of docetaxel 75 mg/m$^2$, doxorubicin 50 mg/m$^2$, and cyclophosphamide 500 mg/m$^2$ (TAC), on day 1, every 3 weeks. Clinical response was determined preferably by sonography or another clinical method if the investigator considered this more appropriate. Early responders were randomized to either four or six cycles of TAC, and nonresponders to either four cycles of TAC or four cycles of vinorelbine 25 mg/m$^2$ on days 1 and 8 plus capecitabine 1,000 mg/m$^2$ orally twice a day on days 1to 14, every 3 weeks. No patient received trastuzumab during neoadjuvant or adjuvant treatment. With a median of 28 days after the beginning of the last chemotherapy cycle, patients underwent surgery. Those with progression were excluded from randomization and treated at the investigator's discretion. Postoperative radiotherapy and endocrine treatment was given according to national guidelines.

### Methods

The study was conducted according to the REporting of tumor MARKer Studies (REMARK) guideline (23) and a prospectively written research, pathologic evaluation, and statistical analysis plan. Ki67 was assessed blinded to the clinical data by immunohistochemistry on the Ventana Discovery autostainer (Ventana) using the antibody MIB-1 as described previously (24). A representative area of the tumor bed containing residual invasive tumor cells was identified. Cells were exactly counted using two manual infactory counting devices (Buggingen, Germany) counting the total cells with one hand and the positive cells with the other hand. In the standard approach, a total of 200 invasive tumor cells were counted in a representative area. In cases

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with minimal residual disease in which the tumor area contained less than 200 cells, all cells present were counted. In a parallel project, the pretherapeutic core biopsies were evaluated for Ki67, as well. The results of the core biopsies are reported in an accompanying publication (24).

The percentage of Ki67-positive cells to the total number of evaluated cells was calculated. Ki67 levels were grouped to low (0–15% stained cells), intermediate (15.1–35% stained cells), and high (>35% stained cells) according to cut-off findings carried out previously for pretreatment Ki67 measurement among the same study population (24).

pCR was defined as no residual invasive disease in any excised breast tissue irrespective of nodal involvement (ypT0/is ypN0/$^{+}$). This differs from previous publications on this study (4, 5, 24) as these are the patients where measurement of Ki67 at the surgical specimen is per se not possible. Histologic response was evaluated locally but the pathology reports were centrally reviewed.

Positive ER and/or PR status was defined as 10% or more positively stained cells or a Remmele intensity and positivity score of 3 or more (25). HER2 status was assessed by immunohistochemistry (positive if 3+) or *in-situ* hybridization.

Survival was defined as the interval between start of TAC chemotherapy and occurrence of a first event. All invasive relapses and all deaths were considered for invasive disease-free survival (IDFS) and OS, respectively, as recommended by Hudis and colleagues (26); primary tumor progression during neoadjuvant treatment was not considered an event. Patients without event, withdrawing consent, or lost to follow-up were censored at the date of last contact.

### Statistical analysis

All analyses were conducted on an intent-to-treat basis in randomized patients with either available surgical tissue for Ki67 measurement or with a pCR ($n$ = 1,151; Fig. 1). Differences between those study participants included and not included in this analysis are shown in Supplementary Table S1. Patients subgroups were compared using Kendals Tau-c test for correlation. Time to event outcome parameters were estimated using the Kaplan–Meier product-limit method, and treatment groups were compared using the log-rank test. Cox proportional hazards models were used to calculate hazard ratios (HR); 95% confidence intervals (CI) are provided. Multivariate Cox regression analysis with and without backward selection was used to

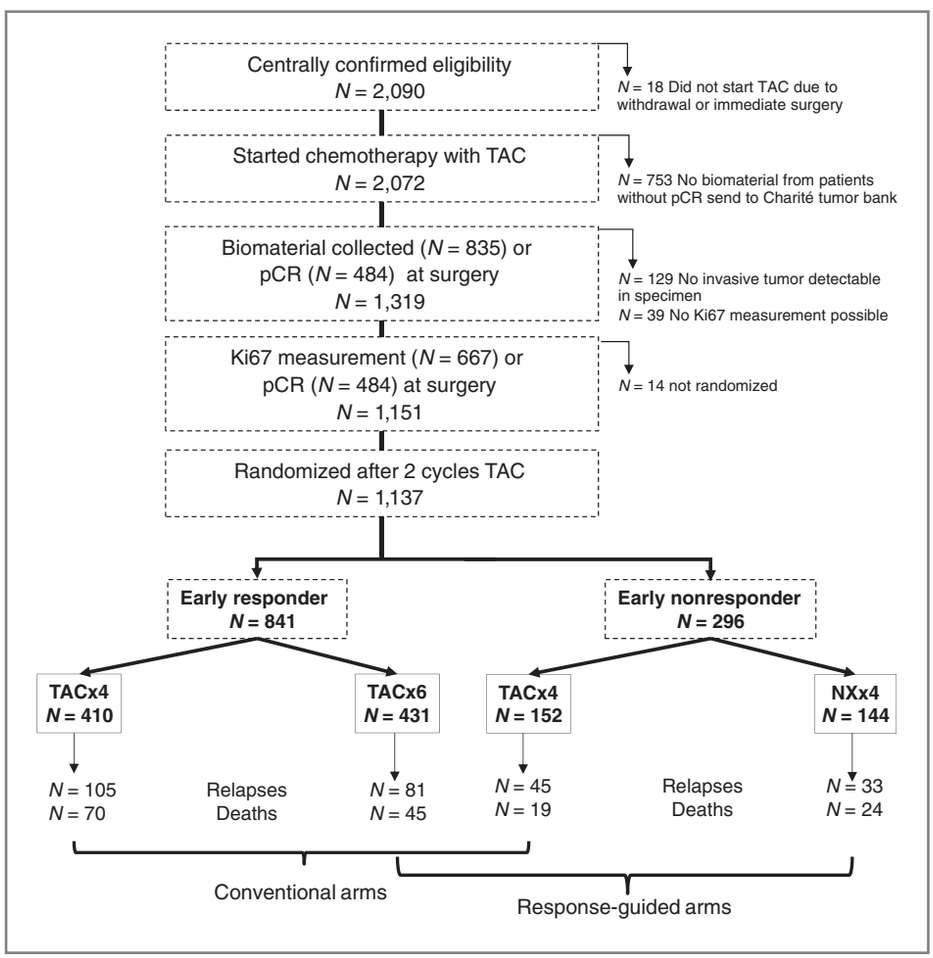


**Figure 1.** Consort statement – patients from the GeparTrio trial included and excluded in the analysis according to availability of tumor material and treatment group. TAC, docetaxel–doxorubicin–cyclophosphamide; pCR, pathologic complete response; NX, vinorelbine–capecitabine.

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**Table 1.** Ki67 levels after neoadjuvant chemotherapy by various patients characteristics

| | Patients with post-treatment Ki-67 measurement or pCR at surgery | | | | |
|---|---|---|---|---|---|
| | pCR (ypT0/is ypN0/$^{+}$) ($n = 484$) | Ki-67 $\leq$15% ($n = 488$) | Ki-67 15.1%–35% ($n = 77$) | Ki-67 >35% ($n = 102$) | Kendals Tau-c test |
| **Characteristic** | ***n* (%)[a]** | ***n* (%)[a]** | ***n* (%)[a]** | ***n* (%)[a]** | ***P* value** |
| Age (yrs) | | | | | <0.0001 |
| <35 | 58 (12.0%) | 20 (4.1%) | 5 (6.5%) | 9 (8.8%) | 0.441[b] |
| 35–<40 | 67 (13.8%) | 40 (8.2%) | 3 (3.9%) | 13 (12.7%) | |
| 40–<50 | 171 (35.3%) | 161 (33.0%) | 27 (35.1%) | 26 (25.5%) | |
| 50–<60 | 115 (23.8%) | 142 (29.1%) | 27 (35.1%) | 26 (25.5%) | |
| 60+ | 73 (15.1%) | 125 (25.6%) | 15 (19.5%) | 28 (27.5%) | |
| Clinical tumor stage | | | | | <0.0001 |
| cT1 | 7 (1.5%) | 6 (1.2%) | 2 (2.6%) | 0 (0.0%) | 0.059[b] |
| cT2 | 357 (74.1%) | 319 (65.4%) | 44 (57.1%) | 62 (60.8%) | |
| cT3 | 73 (15.1%) | 107 (21.9%) | 16 (20.8%) | 24 (23.5%) | |
| cT4a–c | 26 (5.4%) | 41 (8.4%) | 10 (13.0%) | 9 (8.8%) | |
| cT4d | 19 (3.9%) | 15 (3.1%) | 5 (6.5%) | 7 (6.9%) | |
| missing | 2 | 0 (0.0%) | 0 | 0 | |
| Tumor size | | | | | 0.010 |
| <40 mm | 206 (43.1%) | 199 (41.2%) | 26 (33.8%) | 28 (27.5%) | 0.005[b] |
| ≥40 mm | 272 (56.9%) | 284 (58.8%) | 51 (66.2%) | 74 (72.5%) | |
| Missing | 6 | 5 | 0 | 0 | |
| Clinical nodal status | | | | | 0.098 |
| cN negative | 216 (45.3%) | 233 (48.5%) | 32 (41.6%) | 28 (27.5%) | 0.0001[b] |
| cN positive | 261 (54.7%) | 247 (51.5%) | 45 (58.4%) | 74 (72.5%) | |
| Missing | 7 | 8 | 0 | 0 | |
| Tumor type | | | | | 0.221 |
| Ductal invasive | 400 (82.8%) | 375 (76.8%) | 63 (81.8%) | 82 (80.4%) | 0.540[a] |
| Lobular invasive | 33 (6.8%) | 85 (17.4%) | 8 (10.4%) | 5 (4.9%) | |
| Other | 50 (10.4%) | 28 (5.7%) | 6 (7.8%) | 15 (14.7%) | |
| Missing | 1 | 0 | 0 | 0 | |
| Tumor grade | | | | | <0.0001 |
| I | 8 (1.9%) | 31 (6.5%) | 3 (3.9%) | 0 (0.0%) | <0.0001[b] |
| II | 179 (42.1%) | 325 (68.0%) | 47 (61.8%) | 42 (42.0%) | |
| III | 238 (56.0%) | 122 (25.5%) | 26 (34.2%) | 58 (58.0%) | |
| Missing | 59 | 10 | 1 | 2 | |
| HR status | | | | | <0.0001 |
| Positive | 184 (38.0%) | 412 (84.4%) | 57 (74.0%) | 38 (37.3%) | <0.0001[b] |
| Negative | 300 (62.0%) | 76 (15.6%) | 20 (26.0%) | 64 (62.7%) | |
| HER2 status | | | | | 0.001 |
| Negative | 267 (66.3%) | 308 (75.7%) | 50 (78.1%) | 67 (76.1%) | 0.802[b] |
| Positive | 136 (33.7%) | 99 (24.3%) | 14 (21.9%) | 21 (23.9%) | |
| Missing | 81 | 81 | 13 | 14 | |
| HR/HER2 subgroups | | | | | <0.0001 |
| HR$^{+}$/HER2$^{-}$ | 103 (26.4%) | 270 (66.7%) | 42 (65.6%) | 22 (25.0%) | <0.0001[b] |
| HR+/HER2+ | 62 (15.9%) | 76 (18.8%) | 8 (12.5%) | 12 (13.6%) | |
| HR−/HER2+ | 157 (40.3%) | 38 (9.4%) | 8 (12.5%) | 45 (51.1%) | |
| HR−/HER2− | 68 (17.4%) | 21 (5.2%) | 6 (9.4%) | 9 (10.2%) | |
| Missing | 94 | 83 | 13 | 14 | |
| Pretreatment Ki67 | | | | | <0.0001 |
| Low (0%–15%) | 31 (11.8%) | 168 (45.5%) | 15 (28.8%) | 6 (8.2%) | <0.0001[b] |
| Intermediate (15.1%–35%) | 79 (30.0%) | 139 (37.7%) | 19 (36.5%) | 22 (30.1%) | |
| High (>35%) | 153 (58.2%) | 62 (16.8%) | 18 (34.6%) | 45 (61.6%) | |
| Missing | 221 | 119 | 25 | 29 | |

*(Continued on the following page)*

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**Table 1.** Ki67 levels after neoadjuvant chemotherapy by various patients characteristics (Cont'd)

| | Patients with post-treatment Ki-67 measurement or pCR at surgery | | | | |
|---|---|---|---|---|---|
| | pCR (ypT0/is ypN0/$^{+}$) ($n = 484$) | Ki-67 $\leq$15% ($n = 488$) | Ki-67 15.1%–35% ($n = 77$) | Ki-67 >35% ($n = 102$) | Kendals Tau-c test |
| **Characteristic** | ***n* (%)[a]** | ***n* (%)[a]** | ***n* (%)[a]** | ***n* (%)[a]** | ***P* value** |
| Posttreatment T stage | | | | | <0.0001 |
| ypT0/is | 484 (100.0%) | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) | <0.167[b] |
| ypT1 | 0 (0.0%) | 264 (54.1%) | 39 (50.6%) | 52 (51.0%) | |
| ypT2 | 0 (0.0%) | 167 (34.2%) | 23 (29.9%) | 33 (32.4%) | |
| ypT3 | 0 (0.0%) | 47 (9.6%) | 12 (15.6%) | 9 (8.8%) | |
| ypT4 | 0 (0.0%) | 10 (2.0%) | 3 (3.9%) | 8 (7.8%) | |
| Posttreatment N stage | | | | | <0.0001 |
| ypN0 | 421 (87.3%) | 238 (50.1%) | 38 (50.0%) | 47 (0.47) | <0.205[b] |
| ypN1 | 44 (9.1%) | 142 (29.9%) | 16 (21.1%) | 22 (0.22) | |
| ypN2 | 10 (2.1%) | 73 (15.4%) | 15 (19,7%) | 20 (0.20) | |
| ypN3 | 7 (1.5%) | 22 (4.6%) | 71 (93.4%) | 10 (0.10) | |
| Missing | 2 | 13 | 1 | 3 | |
| yAJCC stage | | | | | <0.0001 |
| 0 | 346 (100.0%) | 0 (0.0%) | 32 (15.2%) | 10 (3.8%) | <0.0001[b] |
| 1 | 0 (0.0%) | 154 (75.9%) | 134 (63.5%) | 174 (66.2%) | |
| 2 | 0 (0.0%) | 22 (10.8%) | 18 (8.5%) | 35 (13.3%) | |
| 3 | 0 (0.0%) | 27 (13.3%) | 27 (12.8%) | 44 (16.7%) | |
| Treatment strategy | | | | | 0.965 |
| Conventional | 234 (41.6%) | 246 (43.8%) | 30 (5.3%) | 52 (9.3%) | 0.389[b] |
| Response-guided | 246 (42.8%) | 234 (40.7%) | 46 (8.0%) | 49 (8.5%) | |
| Not randomized | 4 | 8 | 1 | 1 | |
| *HR-positive tumors only* | | | | | 0.955 |
| Conventional | 86 (25.6%) | 209 (62.2%) | 22 (6.5%) | 19 (5.7%) | 0.173[a] |
| Response-guided | 97 (28.0%) | 196 (56.5%) | 35 (10.1%) | 19 (5.5%) | |
| *HR-negative tumors only* | | | | | 0.968 |
| Conventional | 148 (65.5%) | 37 (16.4%) | 8 (3.5%) | 33 (14.6%) | 0.751[a] |
| Response-guided | 149 (65.4%) | 38 (16.7%) | 11 (4.8%) | 30 (13.2%) | |

Abbreviation: HR, hormone receptor.

[a]Valid percentage; for baseline characteristics, percentages are calculated vertically per Ki67 level groups; for treatment strategies, percentages are calculated horizontally between Ki-67 level groups. *P* value compares all patients with Ki-67 measurement or pCR at surgery.

[b]*P* value compares all patients with Ki-67 measurement.

compare the prognostic impact of baseline and surgical variables using the same subgroups as in a previous analysis (27). Variables with *P* values greater than 0.01 were removed stepwise from the model. Subpopulation Treatment Effect Pattern Plot (STEPP; ref. 28) methodology was employed to further illustrate the relationship between Ki67 levels at surgery and outcome across the continuum of Ki67 percentage levels. The STEPP method uses a sliding-window approach to define several overlapping subpopulations of patients. Groups were defined by Ki67 levels of 0% (pCR): 0–10%, 0–20%, 0–30%, 0–40%, 10–50%, 20–60%, 30–70%, 40–80%, 50–90%, and 60–100%. The values on the *x*-axis are the median values of Ki67 levels for patients in these subpopulations, and the *y*-axis indicates the treatment effects, expressed as the Kaplan–Meier estimates of mean IDFS. Each subpopulation contains at least 50 patients and slides by approximately 10% Ki67 levels. All statistical tests were two-sided by default, and *P* values unadjusted for multiple comparisons.

## Results

Posttreatment Ki67 was measured on residual tumor tissue at surgery in 667 patients with available tissue and no pCR out of 2,072 patients that started study treatment (Fig. 1). In addition, 484 patients were included in the analysis that had a pCR at surgery such that assessment of Ki67 level was not possible. Notably, patients with pCR are overrepresented as no surgical tissue collection was required. Of these 1,151 patients, representing 56% of the initial trial population, 562 patients were treated by conventional six cycles TAC, 575 patients with response-guided eight cycles TAC or TAC-NX, and

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14 patients were not randomized. During a median follow-up of 5.2 years, 264 relapses and 158 deaths were observed.

For the 667 patients with available tumor tissue, post-treatment Ki67 was low (0% to 15%) in 488 patients, intermediate (15.1% to 35%) in 77 patients, and high

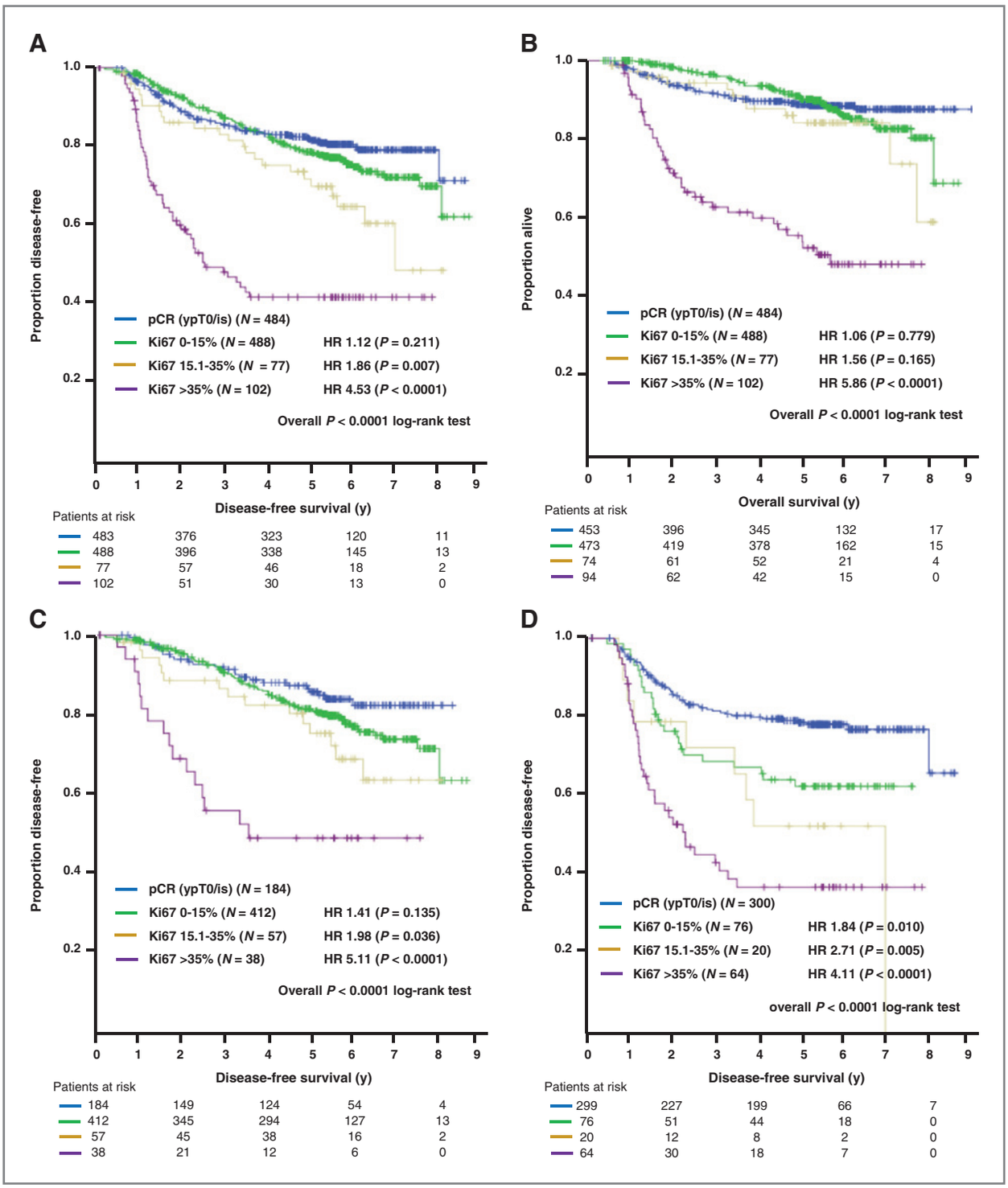


Figure 2. Kaplan–Meier estimates for survival according to Ki67 levels at surgery or change of Ki67 levels from before to after chemotherapy. Disease-free survival (A) and overall survival (B) by Ki67 levels at surgery in all patients; disease-free survival by posttreatment Ki67 levels in patients with hormone–receptor-positive (C) and hormone–receptor-negative (D) disease.

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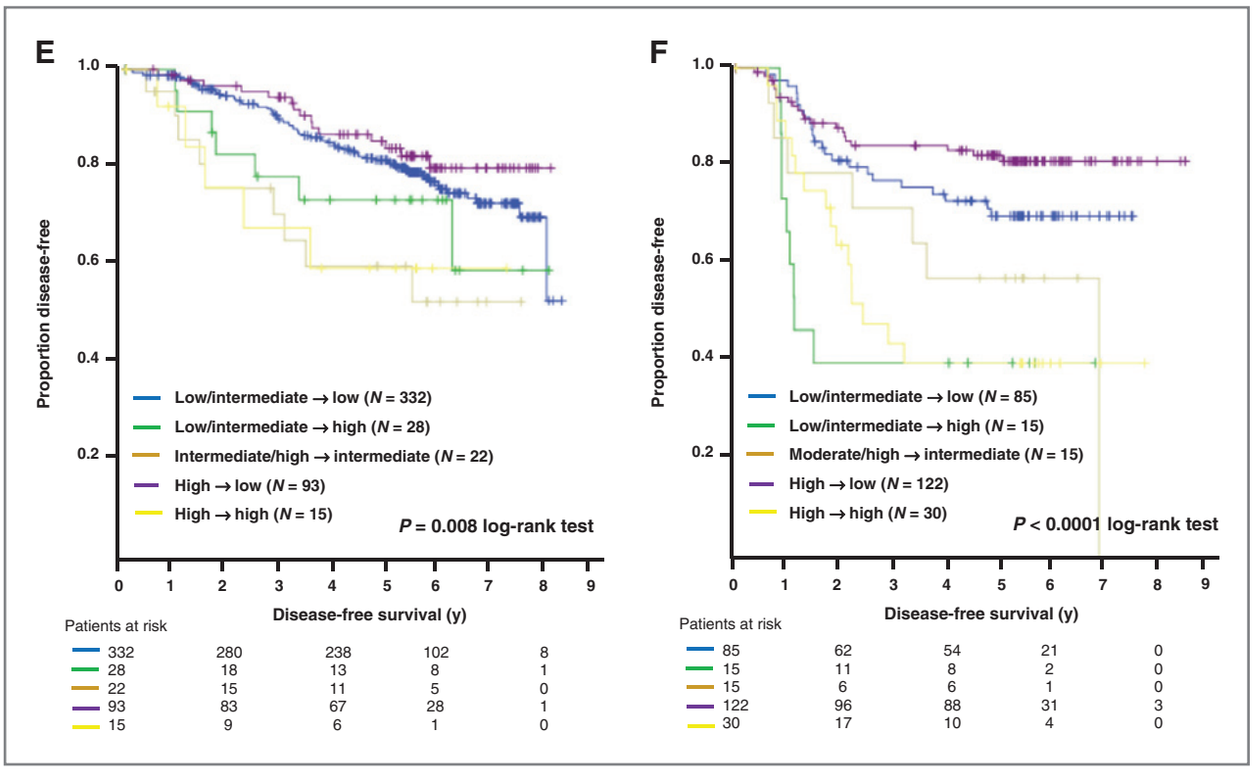


Figure 2. (*Continued*) Disease-free survival by change of Ki67 levels from before to after chemotherapy in patients with hormone–receptor-positive (E) and hormone–receptor-negative (F) disease. Hazard ratios (HR) and *P* values in A–D compare each posttreatment Ki67 level group with the pCR group.

(>35%) in 102 patients (Table 1). Posttreatment Ki67 levels were higher in tumors with a diameter of 40 mm or more, node-positive disease, undifferentiated tumors, hormone–receptor-negative tumors, and tumors with high pretreatment Ki67 levels than in tumors of the corresponding opposite groups.

Patients with high posttreatment Ki67 levels showed higher risk for disease relapse ($P < 0.0001$) and death ($P < 0.0001$) compared with patients having low or intermediate Ki67 levels (Fig. 2A and B). More than 60% of these patients with high posttreatment Ki67 levels suffered from a relapse during the first 3 years after surgery; however, no further relapse occurred after 3 years. Patients with low posttreatment Ki67 levels showed a favorable outcome comparable with patients with a pCR. Patients with intermediate Ki67 levels showed an intermediate risk for relapse, but a risk for death more comparable with the cohort having low Ki67 levels.

Subdividing patients according to the hormone-receptor status of the tumor (Fig. 2C and D) revealed that, in the hormone–receptor-positive cohort, an early and high risk of relapse was observed for those with high posttreatment Ki67 levels. However, for patients with low or intermediate posttreatment Ki67 levels, no obvious difference in disease-free survival to patients with pCR was observed. In the cohort of hormone–receptor negative tumors, patients with low or intermediate posttreatment Ki67 levels showed a higher risk for relapse compared with patients with a pCR, but a lower risk when compared with patients having a high posttreatment Ki67.

In addition, we had paired samples (pre- and postchemotherapy) of 490 patients with hormone–receptor-positive and 267 patients with hormone–receptor-negative tumors. Examining changes of Ki67 from before-to-after neoadjuvant chemotherapy showed that patients with low posttreatment Ki67 levels or pCR had a more favorable outcome irrespective of pretreatment Ki67 levels (Fig. 2E and F). Indeed, patients with a decrease from high pretreatment levels to low posttreatment levels or pCR showed a more sustained low relapse rate after 3 years compared with those patients with low or intermediate levels right from the beginning. Patients with intermediate or high posttreatment Ki67 levels showed a higher relapse rate irrespective of pretreatment Ki67 levels at baseline. This pattern appeared similar in patients with hormone–receptor-negative ($P < 0.0001$) and hormone–receptor-positive tumors ($P = 0.008$).

Ki67 measurement at surgery appeared to provide prognostic information in addition to pCR in most breast cancer subtypes with HRs of 1.64 on average (Fig. 3). Highest HRs among pCR, low, intermediate, and high posttreatment Ki67 levels were found for patients with hormone–receptor-positive/HER2-negative (HR, 1.90) and triple-negative disease (HR, 2.29). No prognostic information was derived from posttreatment Ki67 measurements in patients with lobular cancers.

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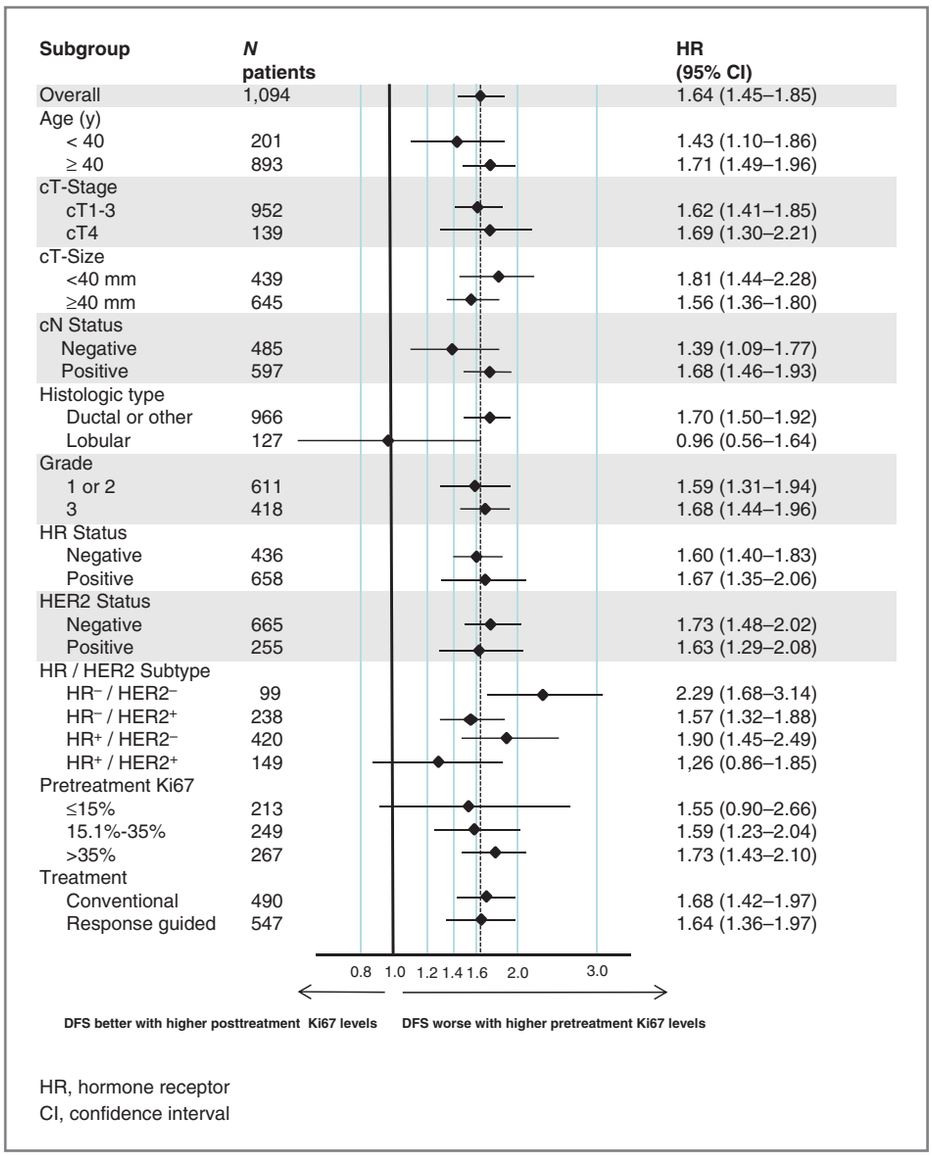


**Figure 3.** Forest plot for the prognostic effect of Ki67 levels at surgery on IDFS in various subgroups. The HR is calculated for differences overall between pCR (ypT0/is), low, intermediate, and high Ki67.

Multivariable Cox regression models examined the additional information deriving from Ki67 measurements at surgery (Table 2). First, a model not including posttreatment Ki67 levels identified confirmation of pCR and histologic nodal status at surgery as well as clinical tumor and nodal stage, hormone-receptor status, and pretreatment Ki67 to provide independent prognostic information for disease-free survival. All other factors (age, histologic tumor type, tumor grade, and HER2 status) of the initial diagnosis were excluded from the model by backward selection. If posttreatment Ki67 levels were included in a second mode, clinical nodal stage, pretreatment Ki67 levels as well as change of Ki67 levels from before to after neoadjuvant chemotherapy were no longer significant prognostic factors. The same Cox models were run separately for patients with hormone–receptor-positive and -negative disease. Although pCR and nodal stage at surgery remained as the only significant predictors of disease-free survival in hormone–receptor-negative disease, full prognostic assessment was possible in hormone–receptor-positive disease by combining posttreatment Ki67 levels and histologic nodal status at surgery, and clinical tumor stage at initial diagnosis.

Patients with conventional and response-guided treatment did not show a different distribution of posttreatment Ki67 levels (Table 1). Mean Ki67 levels were 17.0% after conventional chemotherapy and 16.5% after response-guided chemotherapy. Intermediate Ki67 levels were observed numerically more often after response-guided chemotherapy, in particular in patients with hormone–receptor-positive tumors and in patients after TAC-NX than after conventional chemotherapy (Table 1). Posttreatment Ki67 levels similarly differentiated patients with different

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**Table 2.** Multivariate Cox proportional hazard models on IDFS with or without posttreatment Ki67 levels in all patients as well as in patients with hormone–receptor-positive or -negative tumors

| Parameter[a] | Comparisons | Multivariate model without posttreatment Ki67 ($n = 919$) HR (95% CI) | Multivariate model including posttreatment Ki67 ($n = 609$) HR (95% CI) | Multivariate model including post-treatment Ki67 - HR-positive tumors only ($n = 399$) HR (95% CI) | Multivariate model including post-treatment Ki67 - HR-negative tumors only (N = 210) HR (95% CI) |
|---|---|---|---|---|---|
| Ki67 at surgery | pCR vs. Ki67 0–15% | Not included | 0.93 (0.56–1.56) | 1.82 (0.72–4.61) | 0.61 (.29–127) |
| | 0–15% vs. 15.1–35% | | 1.37 (0.71–2.64) | 2.27 (0.76–6.75) | 1.31 (.52–3.33) |
| | 15.1–35% vs. >35% | | **2.70 (1.54–4.74)** | **5.88 (2.02–17.09)** | 1.73 (.87–3.42) |
| pCR (ypT0 ypN0) | Yes vs. no | **3.75 (2.41–5.86)** | **2.19 (1.19–4.03)** | 1.53 (0.54–4.36) | **4.20 (1.84–9.56)** |
| ypN stage | ypN0 vs. ypN$^+$ | **1.15 (1.10–1.22)** | **1.23 (1.14–1.34)** | **1.21 (1.08–1.35)** | **1.25 (1.10–1.42)** |
| cT stage | cT1-3 vs. cT4a-d | **1.84 (1.41–2.40)** | **2.15 (1.40–2.90)** | **2.07 (1.31–3.27)** | 1.60 (.84–3.05) |
| cN stage | cN0 vs. cN$^+$ | **1.77 (1.39–2.25)** | 1.26 (0.92–1.73) | 1.19 (.79–1.79) | 1.39 (.83–2.31) |
| Hormone-receptor status | Positive vs. negative | **2.12 (1.65–2.73)** | **1.74 (1.24–2.45)** | Not applicable | not applicable |
| Pretreatment Ki67 | 0–15% vs. 15.1–35% | **1.56 (1.13–2.15)** | 1.07 (0.68–1.67) | 0.94 (0.53–1.69) | 1.46 (.69–3.13) |
| | 15.1–35% vs. >35% | **1.14 (.87–1.49)** | 1.30 (0.90–1.87) | 1.33 (0.79–2.26) | 1.16 (.69–1.95) |

Abbreviations: CI, confidence interval; pCR, pathologic complete response.

[a]Age, yAJCC stage, histologic tumor type, tumor grade, and HER2 status were excluded from the model as they did not reach significant results for prediction of disease-free survival.

prognosis after conventional (HR, 1.68) as well as after response-guided (HR, 1.64) chemotherapy (Fig. 3). STEPP analysis investigated differences in disease-free survival of conventional versus response-guided chemotherapy over all posttreatment Ki67 level subgroups (Supplementary Fig. S1). As shown previously, no difference in outcome was observed between the two treatment groups with hormone–receptor-negative disease, which was also the case across all posttreatment Ki67 subgroups. However, patients with hormone–receptor-positive tumors showed the largest differences in mean disease-free survival between conventional and response-guided chemotherapy if posttreatment Ki67 levels were between 20% and 70% in the STEPP analysis.

## Discussion

Centrally assessed nuclear Ki67 expression after neoadjuvant chemotherapy in a subset of 1,151 primary breast cancer patients from a randomized neoadjuvant clinical trial revealed that posttreatment Ki67 adds independent prognostic information surmounting that of pCR regarding the outcome after surgery. However, it appeared that it can provide additional information over pCR, particularly in patients with hormone–receptor-positive disease where the prognostic impact of pCR is limited. We could not show that response-guided systemic treatments led to reduced Ki67 levels at surgery which was the predefined hypothesis. Therefore, this marker cannot improve pCR as a surrogate endpoint marker for neoadjuvant clinical trials in patients with hormone–receptor-positive tumors. However, high posttreatment Ki67 levels identify a group of patients at high risk for relapse, for which additional postsurgical treatment options should be developed.

This is, to the best of our knowledge, the largest cohort on Ki67 measurements after neoadjuvant chemotherapy. As we had information on pre- and posttreatment Ki67 levels available in 757 patients, our analysis had also sufficient statistical power to compare the prognostic impact of pre- and posttreatment Ki67 levels. It appeared that the measurement after neoadjuvant chemotherapy was more important than Ki67 level changes due to treatment. Our findings suggest that tumors with high Ki67 levels at surgery are at high risk of relapse for the first 3 years after diagnosis. This could be explained by these patients had either already high Ki67 levels right at initial diagnosis and were insensitive to treatment or could have predominantly low proliferating, sensitive populations with some high-proliferating, resistant subpopulations, which might have persisted until surgery and were dominant for the prognosis of the patient. Patients with low posttreatment Ki67 levels, irrespective of their pretreatment Ki67 level, showed a relapse risk over the first 3 years comparable with those patients with a pCR; however, the annual relapse risk maintained constant throughout the entire observation period.

Only few studies have reported so far on the prognostic impact of Ki67 measurement after neoadjuvant chemotherapy. The largest, retrospectively collected cohort examined 284 surgical samples and 103 pairs from patients treated at the Royal Marsden Hospital (20), suggesting that Ki67 measured at surgery is a strong predictor of outcome for patients not achieving a pCR. No subgroup analysis by hormone-receptor status was reported. Two recent retrospective cohorts with 102 and 64 patients examined Ki67

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level before and after chemotherapy (17, 19). Although in the larger study only posttreatment Ki67 levels correlated with survival, the smaller study found pre- and posttreatment Ki67 levels and estrogen-receptor status to be correlated with survival. Another report on a cohort of 100 patients has not found any significant changes in Ki67 levels from before to after neoadjuvant chemotherapy (29). Proliferation and estrogen-receptor content are also measured by commercially available gene arrays; however, these tests have only been investigated on pretreatment core biopsies, but not after chemotherapy (30).

Pretreatment Ki67 was measured in two neoadjuvant endocrine studies and an index (preoperative endocrine prognostic index, PEPI) including pathologic tumor size, node status, Ki67 level, and ER status was developed in one trial and validated in the other (31). Patients with a low PEPI score showed a very favorable outcome, not requiring chemotherapy. The PEPI score was similar after treatment with three different aromatase inhibitors in the ACOSOG Z1031 trial (32). However, as these agents show comparable long-term outcome effects, we do not know just how far this score is able to discriminate at short-term a differential long-term efficacy of endocrine agents.

Technical reasons for response-guided chemotherapy not resulting in different posttreatment Ki67 levels might include: (i) the sample size of our analysis might have been too small and was not properly calculated upfront; or (ii) the availability of only 56% of all tumor samples could have biased the results. In addition, we could not investigate effects of postsurgical endocrine treatment; however, this was probably similarly in all patients with hormone–receptor-positive disease. Further, we could have selected a wrong cut-off as none is established for Ki67 (16, 33). However, use of three Ki67 levels should protect from incidental effects observed only with one or the other distinct cut-off. This approach is supported by the observation that mean Ki67 levels were identical between the treatment cohorts.

Central assessment of Ki67 can be considered as a criterion of quality of this work. However, due to high interobserver variability (33, 34), it can be questioned in how far these results can be used in clinical routine. Recent advances of automated image analysis might allow sufficient interlaboratory standardization (35) and might solve this disadvantage of Ki67.

In conclusion, Ki67 levels after neoadjuvant chemotherapy provide relevant independent and additional prognostic information in case of no pCR after neoadjuvant chemotherapy, most notably in hormone–receptor-positive breast cancer. However, posttreatment Ki67 levels were not different after conventional or response-guided chemotherapy. Patients with high posttreatment Ki67 levels are candidates for innovative post-neoadjuvant treatment concepts.

## Disclosure of Potential Conflicts of Interest

Holger Eidtmann has received honoraria from Roche; Wolfgang Eiermann received honoraria from Sanofi, Roche, and Genomic Health; Bernd Gerber has received honoraria from GSK and Astra Zeneca; Jens Huober has received honoraria from Sanofi Aventis, Roche, and BMS; Tanja Fehm has received honoraria from Novartis, Roche, and GSK; Peter A. Fasching has received honoraria from Novartis; Volkmar Müller has received honoraria from Roche, Amgen, Celgene, Sanofi Aventis, and Pierre Fabre, and Christian Jackisch has received honoraria from GSK and Roche. No potential conflicts of interest were disclosed by the other authors.

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