IHC-based Ki67 as response biomarker to tamoxifen in breast cancer window trials enrolling premenopausal women

Stacey E. P. Joosten1, Marius Wellenstein2, Rutger Koornstra3, Annelot van Rossum4, Joyce Sanders5, Vincent van der Noort6, Maria C. Ferrandez5, Rolf Harke5, Ingrid A. M. Mandjes6, Hilde Rosing7, Alwin Huitema7,8,9, Jos H. Beijnen7,8,10, Jelle Wesseling5,11, Paul J. van Diest12, Hugo M. Horlings13, Sabine C. Linn4,12,13,5 and Wilbert Zwart1,14.

INTRODUCTION

Presurgical window studies are gaining traction to assess (molecular) changes in short timeframes. Decreased tumor cell positivity for the proliferation marker Ki67 is often used as a proxy for treatment response. Immunohistochemistry (IHC)-based Ki67 on tissue from neoadjuvant trials was previously reported to be predictive for long-term response to endocrine therapy for breast cancer in postmenopausal women, but none of these trials enrolled premenopausal women. Nonetheless, the marker is being used on this subpopulation. We compared pathologist assessed IHC-based Ki67 in samples from pre- and postmenopausal women in a neo-adjuvant, endocrine therapy focused trial (NCT00738777), randomized between tamoxifen, anastrozole, or fulvestrant. These results were compared with (1) IHC-based Ki67 scoring by AI, (2) mitotic figures, (3) mRNA-based Ki67, (4) five independent gene expression signatures capturing proliferation, and (5) blood levels for tamoxifen and its metabolites as well as estradiol. Upon tamoxifen, IHC-based Ki67 levels were decreased in both pre- and postmenopausal breast cancer patients, which was confirmed using mRNA-based cell proliferation markers. The magnitude of decrease of Ki67 IHC was smaller in pre- versus postmenopausal women. We found a direct relationship between post-treatment estradiol levels and the magnitude of the Ki67 decrease in tumors. These data suggest IHC-based Ki67 may be an appropriate biomarker for tamoxifen response in premenopausal breast cancer patients, but anti-proliferative effect size depends on estradiol levels.

RESULTS

IHC-based Ki67 decrease differs between treatment arms

Postmenopausal patients with primary, estrogen receptor-positive (ER+) breast cancer were randomized to several weeks of tamoxifen, anastrozole, or fulvestrant prior to routine surgery, while all premenopausal patients received tamoxifen by default. Tumor material was collected before and after treatment (Fig. 1). We therefore analyzed the performance of Ki67 staining in pre-versus postmenopausal breast cancer patients treated with tamoxifen, enrolled in a neo-adjuvant, endocrine therapy study by comparing the change in IHC-based Ki67 assessed by pathologists and artificial intelligence to gene expression-based Ki67, gene expression signatures capturing proliferation, mitotic figure counts as well as blood levels of tamoxifen or its metabolites and estradiol.
contour (Fig. 2a). This resulted in an algorithm with
colour and brightness of the staining area within each nucleus
In short, Ki67 staining positivity in tumor cells is determined by the
Institute between 2010
4599 breast cancer patients treated at the Netherlands Cancer
containing whole slide images of KI67 stained tumor tissue of
algorithm was trained and validated on a dataset
with WSK medical, by means of a convolutional neural network
with ≥80% decrease only considering
0.035). A larger effect size was observed for postmenopausal
women with ~63% decrease when considering all patients (or
~80% decrease only considering “responders”) versus ~35%
decrease in premenopausal patients (or ~60% decrease when
only considering “responders”) (Fig. 3b). In postmenopausal
patients, the magnitude of treatment effect on Ki67 did not
statistically differ between tamoxifen, anastrozole, and fulvestrant
(p-value = 0.057), also not when only considering “responders”,
which may be impacted by the small sample size in these groups.
To assess whether all tamoxifen-treated patients actually took
the drug, serum levels of tamoxifen and metabolites thereof were
measured in blood samples taken prior to surgery (Supplementary
Fig. 3b). Each patient had detectable tamoxifen values. No
differences were found for tamoxifen levels itself between pre-
and postmenopausal patients, nor for any of the metabolites we
analyzed. None of these variables correlated with the magnitude
of the Ki67 change. Duration of treatment, BML, tumor grade-
and histological type did not statistically differ between menopausal
status in tamoxifen-treated patients for whom algorithm-assessed
Ki67 levels were available (Supplementary Table 1). Treatment
duration also did not correlate with the magnitude of effect, when
stratified for menopausal status. Lymph node involvement did
differ, but even among LN negative patients who received
tamoxifen, the magnitude of Ki67 change remained significantly
different based on menopausal status. Post-treatment IHC-based
PR values (as determined by a pathologist) were slightly higher in
postmenopausal patients (p-value = 0.001) (Supplementary Table
1), but the effect on PR levels due to tamoxifen was of similar
magnitude (p-value = NS) in pre- and postmenopausal patients. We
hypothesized that competition of tamoxifen with estradiol,
the latter being decreased in menopause, might underline the
difference in treatment effect. We therefore measured estradiol
levels (E2) in the blood of patients. For most postmenopausal
women, pretreatment E2 levels fell below the detection limit of
44 pmol/L. In premenopausal women, we noted both increases as
well as decreases in E2 levels when analyzing pre- and post-
treatment E2 measurements (Supplementary Fig. 3a). When we
examined the gradient of post-treatment E2 measurements in
tamoxifen-treated patients with E2 levels above the detection
limit, we found a significant inverse association (p-value = 0.004)
between the effect size in Ki67 and post-treatment E2 levels (Fig.
3c). Thus, high estradiol levels may compete with tamoxifen to
dampen the treatment effect on tumor cell proliferation.
Gene expression-based Ki67 and proliferation signatures illustrate general decrease of cell proliferation signaling for all treatment arms

Based on the pathological assessment, we found 50% of premenopausal patients to not show a decrease in Ki67 upon tamoxifen treatment. However, assessment by an AI algorithm did not support this. Both methods agreed in the observation that the decrease of Ki67 upon tamoxifen treatment was of lower magnitude for pre-menopausal patients. To provide an independent quantifiable readout of cellular proliferation signaling in relation to treatment response, we generated gene expression data for all arms, as described previously.

In almost all cases, mRNA for Ki67 decreased upon treatment (Fig. 4a). When we analyzed the Ki67 mRNA data for a potentially larger anti-proliferative effect in postmenopausal women who received tamoxifen, versus premenopausal patients, we confirmed a statistically significant difference (p-value<0.04). To confirm treatment effect on cell proliferation by yet other means, we calculated five independent, previously reported gene expression-based proliferation signatures (Fig. 4b and c, Supplementary Fig. 4). AURKA signature indicated proliferation decreased statistically significantly in all treatment groups, as did CIN70 and GGI. However, GENE70 scores before and after treatment did not change significantly for premenopausal, nor did E2F3 for anastrozole. Generally, concordance between proliferation signatures was high and concordance between Ki67 mRNA expression and other proliferation signatures (i.e., AURKA) was high. However, in an unexpectedly large number of samples, the Ki67 mRNA expression showed a significant divergence from the proliferation signatures (Supplementary Fig. 4).

Table 1. Baseline characteristics of all patients randomized in the trial and eligible for analysis.

|                      | Premenopausal | Postmenopausal | P values |
|----------------------|---------------|----------------|----------|
|                      | tamoxifen     | tamoxifen      | anastrozole | fulvestrant | tamoxifen pre-versus tamoxifen postmenopausal arms |
|                      | (n = 35)      | (n = 18)       | (n = 17)   | (n = 19)    |                                              |
| Age at inclusion     | 46.7 ± 0.8    | 61.5 ± 1.8     | 62.9 ± 2.0 | 61.8 ± 2.1  | p < 0.001 p = NS p = NS                     |
| BMI                  | 25.2 ± 0.9    | 26.7 ± 0.9     | 26.8 ± 1.4 | 28.6 ± 1.4  | p = NS p = NS p = NS                       |
| Hospital             | NKI           | Nijmegen       |           |             |                                              |
|                      | 32 (91.4%)    | 3 (15.8%)      | 4 (23.5%)  | 3 (16.7%)   |                                              |
| Treatment duration   | 23.8 ± 1.7    | 17.4 ± 1.9     | 19.5 ± 2.3 | 21.2 ± 3.0  | p = 0.011 p = NS                           |
| in days (Start HT – OR) |              |                |            |             |                                              |
| Histopathology       |               |                |            |             |                                              |
| Laterality           | Right         | Left           |           |             |                                              |
|                      | 19 (54.3%)    | 8 (42.1%)      | 5 (29.4%)  | 9 (50.0%)   | p = NS p = NS                              |
|                      | 16 (45.7%)    | 9 (47.4%)      | 12 (70.6%) | 9 (50.0%)   |                                              |
|                      | 0 (0 %)       | 2 (10.5%)      | 0 (0%)     | 0 (0%)      |                                              |
| Bilateral            |               |                |            |             |                                              |
| Histology            | Ductal        | Lobular        | Mixed Ductal/Lobular | Other (Mucinous/ Tubular/ Apocrine) |                                              |
|                      | 24 (68.6%)    | 6 (17.1%)      | 2 (5.7%)   | 2 (5.7%)    |                                              |
|                      | 17 (89.5%)    | 13 (76.5%)     | 0 (0%)     | 0 (0%)      |                                              |
|                      |               | 3 (17.6%)      | 0 (0%)     | 1 (5.6%)    | p = NS p = NS                              |
|                      |               | 3 (17.6%)      |            | 3 (11.1%)   |                                              |
| Differentiation grade| Good          | Moderate       | Poor       | Not assessed |                                              |
|                      | 11 (31.4%)    | 15 (42.9%)     | 4 (11.4%)  | 4 (11.4%)   | p = NS p = NS                              |
|                      | 7 (36.8%)     | 11 (57.9%)     | 1 (5.3%)   | 2 (10.5%)   |                                              |
|                      |               | 9 (52.9%)      | 2 (11.8%)  | 3 (17.6%)   | p = NS p = NS                              |
|                      |               | 9 (50%)        | 2 (11.1%)  | 3 (16.7%)   |                                              |
| Tumor size in mm     | 15.4 ± 1.6    | 18.4 ± 3.2     | 15.9 ± 2.6 | 17.6 ± 2.0  | p = NS p = NS                              |
|                      | (n = 31)      | (n = 18)       | (n = 18)   | (n = 18)    |                                              |
| Type of surgery      | Mastectomy    | Wide Local Excision | Biopsy | None |                                              |
|                      | 14 (40.0%)    | 17 (48.6%)     | 3 (8.6%)   | 4 (11.4%)   |                                              |
|                      | 7 (36.8%)     | 11 (57.9%)     | 1 (5.3%)   | 2 (10.5%)   | p = NS p = NS                              |
|                      |               | 10 (58.8%)     | 4 (23.5%)  | 3 (17.6%)   |                                              |
|                      |               | 12 (66.7%)     | 0 (0%)     | 3 (11.1%)   | p = NS p = NS                              |
|                      |               | 6 (33.3%)      | 0 (0%)     | 3 (16.7%)   |                                              |
|                   |               |               |            |             |                                              |
| LN involvement       | Negative      | (sub)micrometastases | Positive | NA or ND |                                              |
|                      | 25 (71.4%)    | 7 (20.0%)      | 0 (0.0%)   | 2 (5.8%)    |                                              |
|                      | 12 (63.2%)    | 2 (10.5%)      | 0 (0.0%)   | 2 (10.5%)   |                                              |
|                      | 11 (64.7%)    | 1 (5.9%)       | 0 (0.0%)   | 3 (17.6%)   |                                              |
|                      | 12 (66.7%)    | 2 (11.1%)      | 0 (0.0%)   | 3 (5.6%)    | p = NS p = NS                              |
|                   |               |               |            |             |                                              |
| IHC on pre treatment biopsy |               |               |            |             |                                              |
| IHC ER in %          | 89.4 ± 3.2    | 98.8 ± 1.2     | 92.5 ± 3.9 | 97.5 ± 1.0  | p = 0.004 p = NS                           |
|                      | (n = 33)      | (n = 17)       | (n = 16)   | (n = 16)    |                                              |
| IHC PR in %          | 58.9 ± 6.2    | 66.4 ± 8.1     | 49.0 ± 11.2| 63.9 ± 9.1  | p = NS p = NS                              |
|                      | (n = 32)      | (n = 18)       | (n = 16)   | (n = 14)    |                                              |
| IHC HER2 in %        | 33 (94.3%)    | 18 (94.7%)     | 14 (82.4%) | 15 (83.3%)  | NA p = NS                                  |
|                      | (n = 33)      | (n = 17)       | (n = 16)   | (n = 16)    |                                              |
|                  | Positive       |               |            |             |                                              |
|                      | 0 (0 %)       | 0 (0%)         | 0 (0%)     | 2 (11.8%)   |                                              |
|                      | 2 (11.8%)     | 0 (0%)         | 2 (11.8%)  | 5 (5.6%)    | p = NS p = NS                              |
| IHC Ki67 in %        | 15.2 ± 2.7    | 17.1 ± 4.1     | 11.0 ± 1.9 | 9.3 ± 2.4   | p = NS p = NS                              |
|                      | (n = 33)      | (n = 15)       | (n = 16)   | (n = 16)    |                                              |

Categorical variables are displayed as frequencies and corresponding percentages within the treatment group and p values resulted from two-sided Fisher’s exact tests. Continuous variables are displayed as mean value ± SEM (n) and p values resulted from Mann Whitney U tests when comparing pre-versus postmenopausal patients randomized to tamoxifen or Kruskal–Wallis tests when comparing all postmenopausal arms. Two-sided t-test and ANOVA (unadjusted p value displayed) were performed on log (Ki67 + 1) values. NS = not significant.
As a third independent method to assess cell proliferation, mitotic figures were counted on Hematoxylin and Eosin slides of resection material. In agreement with all above-mentioned readouts, no difference in their number was observed in pre-treatment or post-treatment samples, for all treatment groups (Supplementary Fig. 5).

In general, we found that premenopausal patients as well as postmenopausal patients show a decrease in artificial intelligence algorithm assessed- and mRNA-based Ki67 levels upon tamoxifen treatment in the neo-adjuvant setting. Though, on the basis of both these measurements, the decrease in Ki67 levels was slightly less in premenopausal patients. We found a significant relationship between the magnitude of decrease in Ki67 and patients E2 levels after treatment, which would fit with the biology of tamoxifen competing with estradiol over the binding of the estrogen receptor in these ER+ tumors. Other methods to assess proliferation, such as proliferation signatures and mitotic figure counts, imply that premenopausal patients indeed respond to tamoxifen, but to a similar degree as postmenopausal women.

Taked together, these results imply that IHC-based Ki67, when assessed with rigor, maybe an appropriate biomarker for the premenopausal breast cancer patient to assess tumor response to tamoxifen, on a group level.

DISCUSSION

IHC-based Ki67 was previously studied and validated as a surrogate endpoint for treatment response to endocrine therapy for ER+ breast cancer. However, none of the postmenopausal trials included a 40 mg tamoxifen loading dose schedule, required to reach steady-state levels within a week7–10,18. In addition, these studies were limited to tamoxifen and AI treatment in postmenopausal women. Ki67 is increasingly being used as a response marker for premenopausal women as well, despite a lack of supporting evidence. The number of studies doing so may increase, as the FDA recently issued a guidance document, to encourage the inclusion of more premenopausal women in breast cancer trials that investigate the efficacy of hormonal agents.

We performed a neo-adjuvant trial with postmenopausal women receiving either tamoxifen (including loading dose), anastrozole or fulvestrant, and premenopausal women treated with tamoxifen (including loading dose). Though from a modest number of patients, this dataset allowed us to assess the relationship between response to endocrine treatment on the basis of IHC-based Ki67, within a single patient and per menopausal status, to Ki67 mRNA, proliferation signatures, estradiol levels, tamoxifen metabolites, clinicopathological features, and patient outcome. We were able to confirm, by traditional pathology, that IHC-based Ki67 significantly decreases upon anastrozole treatment, as well as upon tamoxifen treatment in pre- and postmenopausal women. However, in our dataset, initially nearly 50% of premenopausal patients displayed increased or unaltered IHC-based Ki67 levels, which would indicate that these patients did not benefit from the treatment. Increased Ki67 IHC levels in a small subset of patients have been reported in previous studies enrolling postmenopausal patients who received standard-dose tamoxifen10,19, arguing against the use of a tamoxifen loading dose as a possible driver for this increase. There has been much debate on inter- and intra-observer variability in IHC-based Ki67, which might also underline this observation. To more objectively assess the endpoint results of this trial, we employed an artificial intelligence algorithm to detect Ki67 levels on immunohistochemistry slides from patient breast tumors. Using this algorithm, we still found IHC-based Ki67 levels to decrease in both pre- and postmenopausal women who received tamoxifen. Assessment of proliferation by other methods, including 5 proliferation signatures and mitotic figures, also support the use of Ki67 as a biomarker for premenopausal women. However, when examining the decrease in IHC-based Ki67 levels as determined by artificial intelligence levels more closely, we noted that the decrease in these was less apparent for premenopausal women who received tamoxifen, than postmenopausal women who received tamoxifen. After excluding this was caused by clinicopathological features such as tumor histology, stage or lymph node involvement, we assessed the magnitude of effect on Ki67 with regards to the gradient of estradiol levels found in the blood of premenopausal women. We found a significant relationship between the two variables, which may reflect competition of estradiol and tamoxifen for the estrogen receptor driving these ER+ tumors. The difference in magnitude of effect in pre- and postmenopausal women was additionally supported by mRNA data. Whether Ki67 effect size
corresponds with long-term treatment outcome, is yet to be
investigated. This study provides evidence that supports the
added value of artificial intelligence in pathology, and may prove
useful in addressing the known inter-observer variations in
Ki67 scoring, resulting in a highly quantitative measure of Ki67
positivity on IHC. Hence, artificial intelligence may be of use for
other neoadjuvant studies using Ki67 IHC as a biomarker for
treatment response.

On few occasions, we noted that depending on the method to
assess proliferation, a single patient may be considered a "responder"
by one, and a "non-responder" by another method. There may be various reasons that could underlie this, for
example - but not limited to – precision of a method (e.g. variance). This includes IHC-based Ki67, for which precision has
not been investigated. We therefore advise that interpreting
response to therapy in an individual patient should therefore be
done with caution. We would like to stress that IHC-based Ki67
was never put forward in literature as a patient-level biomarker
nor have patient-level interpretations of IHC-based Ki67 been
extensively investigated. Still, clinicaltrials.gov currently lists
several breast cancer window trials that utilize IHC-based Ki67 as a
primary endpoint measure to assess drug response on a patient
level, including those that enroll premenopausal women. In- and
outside the context of such trials, patient-level IHC-based Ki67 is
used as a measure for endocrine therapy sensitivity and thus
treatment guidance. Therefore, we encourage further research
into the use of IHC-based Ki67 as a patient-level marker.

To our knowledge, we show the first evidence that supports
IHC-based Ki67, when assessed rigorously and quantitatively, can be
used as a biomarker to assess tamoxifen response in premeno-
pausal breast cancer patients.

**METHODS**

**Clinical trial**

Between 2008 and 2016, 94 patients with primary, operable, estrogen receptor-positive (ER+) breast cancer (Supplementary Fig. 1a for detailed
criteria) were registered for an open-label, randomized phase-2 trial
(NCT00738777) at the Netherlands Cancer Institute and the Radboud
Medical Centre. The primary objective of the trial was to prospectively
investigate whether short-term endocrine treatment can induce molecular
changes predictive of therapy response. The decrease in proliferation in
this interval, measured by pathologist’s assessment of IHC-based Ki67, was
pre-specified as a primary endpoint. A core needle biopsy of the tumor was
taken prior to treatment. Following treatment, a surgical specimen was
taken. The date of the surgery was determined by standard clinical
guidelines and planning. All premenopausal women received tamoxifen.
Postmenopausal women were randomized to either tamoxifen, anastro-
zeole, or fulvestrant treatment. At the initiation of the trial, fulvestrant
was not given as a monotherapy, but combined with anastrozole. After the
inclusion of six patients in this arm, a protocol was amended to fulvestrant
monotherapy. Dosages were based on previously published studies. Of note, a tamoxifen loading dose of 40 mg bi-daily was given in the first week to reach steady-state levels within the duration of treatment.

Study approval
The clinical trial protocol was approved by the local medical ethics committee of the Netherlands Cancer Institute, in accordance with appropriate international ethical guidelines, and written informed consent was obtained from all patients. The research has been approved by the Netherlands Cancer Institute’s institutional review board.

Immunohistochemistry and pathologist assessment
Immunohistochemistry (IHC) for Ki67 (Ultraview DAB followed by 32 min antibody retrieval and 32 min incubation with MIB M7240, Dako) was performed at a single pathology facility on pre- and posttreatment patient material. Slides were assessed centrally by an experienced breast pathologist by visual inspection of the whole slide. If pathologists indicated a range of percentage positive cells for Ki67, the highest number was used for analyses. Pathologist-assessed IHC-based Ki67 is lognormally distributed and contained some 0 values. T-test and ANOVA were therefore performed on log10 (Ki67 + 1) values. To examine extent of change in pathologist-assessed values of Ki67 across treatment arms, log10 ((posttreatment Ki67 + 1)/(pretreatment Ki67 + 1)) was compared.

Artificial intelligence algorithm
A convolutional neural network (CNN), was used to develop the Ki-67 algorithm, which is based on millions of patches sourced from Whole Slide Images (WSI), supplied by the NKI. A sliding window of 80 x 80 pixels is first applied to the IHC stained image, with a stride of 10 pixels, to generate image tiles. Then the trained machine learning model outputs a probability of a nucleus in the centre of a tile, i.e. a probability map with a grid size of 10 x 10
pixels. Then a Gaussian filter is applied on the probability map to obtain a nucleus map and thus a set of nucleus contours can be obtained from the nucleus map, resulting in the location of positive and negatively coloured nuclei with also the percentage amount of positive-coloured nuclei within the Region Of Interest. With these patches, we developed a CNN to make patch-level predictions to detect Ki-67 positive and negative stained nuclei. The reliability of the algorithm was tested against the ground truth. The ground truth is based on an annotated dataset21 with which we built the initial algorithm. We then expanded the algorithm using data from the NKI Pathology archive, to clarify the nuances in staining intensity (light blue, light gray/blue), and to segment stroma and other “noise” as background. The deep learning algorithm was trained and validated on a dataset containing 4,599 breast cancer tissue WSI supplied from the NKI Pathology archive. Four thousand 80 × 80 pixel patches were then extracted from each WSI, resulting in 18,396,000, 80 × 80 pixel patches. We used 14,716,800 patches for training and 3,679,200 patches for validation.

During model training, the patch-based classification stage takes as input Ki-67 positive WSI containing breast cancer tissue. We randomly extracted millions of small Ki-67 positive and negative patches from the training set. Following the selection of positive and negative training examples, we trained a supervised classification model to discriminate between these two classes of patches.

The staining positivity is determined by the colour and brightness of the area within each nucleus contour. We implemented a sliding window algorithm in order to identify positive and negative nuclei in each region of interest 1 mm² for each slide. A Gaussian filter is also applied on the probability map to obtain a nucleus map. Thus, a set of nucleus contours can be obtained from the nucleus map. The performance of the model was tested on the validation dataset and the percentage of reliability is based on the results from the training against the validation, resulting in a reliability of 92% or higher. The output of the Ki-67 module reports the total number of detected nuclei (positive & negative), the number of Ki-67 positive nuclei and the Ki-67 proliferation index (percentage of the total number of detected nuclei that was positive for Ki-67) within the selected region of interest (ROI).

Tamoxifen, -metabolites and estradiol measurements
Tamoxifen and the five active metabolites N-desmethyltamoxifen, 4-hydroxytamoxifen, 4'-hydroxytamoxifen, N-desmethyl-4'-hydroxytamoxifen (Z-endoxifen) and N-desmethyl-4'-hydroxytamoxifen were quantified in patient serum with a validated liquid chromatography-tandem mass spectrometry bioanalytical method22. Estradiol measurements were performed using a second-generation Cobas Estradiol immunoassay, and run on a Cobas 6000 device from Roche Diagnostics, following the standard manufacturer’s instructions.

Gene expression
RNA was isolated and hybridized to a custom full genome array by Agendia as described previously23. RNA was isolated from FFPE sections from the using the Qiagen RNeasy FFPE kit. 50 ng of total RNA was subsequently transcribed, amplified (Rubicon; C-WTA kit C), labeled with Cy3 (Genomic DNA enzymatic Labeling kit; Agilent Technologies), and purified again (Amicon ultra 30 kDa filters). The labeled cDNA was hybridized to a custom full genome array (based on Agilent Catalog #G2514F) at 65 °C for 17 h, then washed, after which the array was scanned with a dual laser scanner (Agilent Technologies). Feature Extraction software v11.5.1.1 was used to quantify fluorescent intensities and those were normalized using DataPrint software v1.15. Missing values were imputed with kNN 10. RNA was isolated from FFPE sections from the using the Qiagen RNeasy FFPE kit. 50 ng of total RNA was subsequently transcribed, amplified (Rubicon; C-WTA kit C), labeled with Cy3 (Genomic DNA enzymatic Labeling kit; Agilent Technologies), and purified again (Amicon ultra 30 kDa filters). The labeled cDNA was hybridized to a custom full genome array (based on Agilent Catalog #G2514F) at 65 °C for 17 h, then washed, after which the array was scanned with a dual laser scanner (Agilent Technologies). Feature Extraction software v11.5.1.1 was used to quantify fluorescent intensities and those were normalized using DataPrint software v1.15. Missing values were imputed with kNN 10. Data were batch corrected for date of RNA extraction using ComBat from the R package sva, and the median value was used in case multiple probes mapped to a single gene. Statistical analysis to compare mRNA levels of Ki67 (moderated t-test) was performed with Limma v3.37.3 in R. For gene expression signatures, per signature, gene expression data was subsetted to genes required for the respective signature and the signature score was calculated, per sample, as was previously described23.

Mitotic figure counts
An expert pathologist in mitotic figures (PoV) blindly scored excision specimens from all treatment groups, according to published guidelines24. Since the biopsy samples were limited in total surface area and had a poor representation of intra-tumor heterogeneity, only post-treatment resection slides were analysed. A cellular region of 0.5 cm × 0.5 cm was analysed for each tumor sample, in which extrapolation was needed for a limited number of cases, without a preference for a particular treatment arm or menopausal status.

Statistics
For each variable, (normal) distribution was assessed using qqplots and histograms with IBM SPSS Statistics 25. In almost all cases, non-parametric tests were applied or data were log-transformed. Prior to any T-test, Levene’s test for variance was performed. For detailed explanations, per variable, please see legends.

Reporting summary
Further information on research design is available in the Nature Research Reporting Summary linked to this article.

DATA AVAILABILITY
Gene expression data and accompanying clinical parameters are available on the GEO repository (GSE147271). Additional patients parameters can be made available upon request to the corresponding author.

CODE AVAILABILITY
R scripts (run on R version 3.5.1) describing gene expression analysis are available upon request to a corresponding author. Details regarding these analysis, including used packages, are specified in the Methods section. Code underlying Ki67 artificial intelligence algorithm is proprietary to WSK Medical B.V., Amsterdam, The Netherlands.

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AUTHOR CONTRIBUTIONS
SJ acquired and analyzed the data, performed statistics, and facilitated trial logistics. MW and HH were responsible for the development of the artificial intelligence algorithm for Ki67. RK was responsible for the clinical trial setup. AvR was responsible for clinical trial logistics and patient data acquisition. JS reviewed patient material. VvdN consulted on statistics. MH assisted in developing the artificial intelligence algorithm for Ki67. RH supervised MH. IM facilitated trial logistics. HR, AH, and JB were responsible for blood measurements. JW, PvD, and HH reviewed patient material and consulted on methodology. SJ, SL, and WZ conceptualized the research. SJ, SL, and WZ wrote the manuscript and all co-authors provided input and feedback. SL and WZ acquired funding.

COMPETING INTERESTS
The funder had no role in the design of the study; the collection, analysis, and interpretation of the data; the writing of the manuscript; or the decision to submit the manuscript for publication. Fulvestrant was provided by AstraZeneca. Gene expression was generated by Agendia Inc, partly under a service agreement, partly as a collaboration. Marius Wellenstein is Chief Operating Officer for WSK Medical B.V. The remaining authors declare no competing interests.

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Correspondence and requests for materials should be addressed to Hugo M. Horlings, Sabine C. Linn or Wilbert Zwart.
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