Biomarker Analysis of Neoadjuvant Doxorubicin/Cyclophosphamide
Followed by Ixabepilone or Paclitaxel in Early-stage Breast Cancer

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Statement of translational relevance

The neoadjuvant setting provides a unique scenario for identifying biomarkers that are predictive of treatment response in breast cancer patients. Single-agent neoadjuvant ixabepilone has previously demonstrated promising activity in invasive breast cancer, particularly in patients with high βIII-tubulin expression. This randomized, phase II trial evaluated potential biomarkers, including βIII-tubulin, which may differentiate response to neoadjuvant ixabepilone relative to paclitaxel in early-stage breast cancer. No correlation was evident between βIII-tubulin protein and mRNA expression, MDR1 protein expression, TACC3 and CAPG gene expression, and multi-gene expression models (20- and 26-gene), and the efficacy of ixabepilone or paclitaxel, indicating that these markers are not predictive of differentiating treatment benefit in this patient setting. Higher pathologic complete response rates were observed among βIII-tubulin-positive patients compared with βIII-tubulin-negative patients; however, this was true for both the ixabepilone- and paclitaxel-treated cohorts.
Abstract

**Background:** Predictive biomarkers offer the potential to improve the benefit:risk ratio of a therapeutic agent. Ixabepilone achieves comparable pathologic complete response (pCR) rates to other active drugs in the neoadjuvant setting. This phase II trial was designed to investigate potential biomarkers that differentiate response to this agent.

**Patients and methods:** Women with untreated, histologically-confirmed primary invasive breast adenocarcinoma received neoadjuvant doxorubicin/cyclophosphamide, followed by 1:1 randomization to ixabepilone ($n = 148$) or paclitaxel ($n = 147$). Rates of pCR were compared between treatment arms based on pre-defined biomarker sets: $TUBB3$, $TACC3$ and $CAPG$ gene expression, a 20- and 26-gene expression model, MDR1 protein expression and other potential markers of sensitivity. βIII-tubulin protein expression is reported separately, but is referred to here for completeness. All patients underwent a core needle biopsy of the primary cancer for molecular marker analysis prior to chemotherapy. Gene expression profiling data was used for molecular subtyping.

**Results:** There was no significant difference in the rate of pCR in both treatment arms in βIII-tubulin-positive patients. Higher pCR rates were observed among βIII-tubulin-positive patients compared with βIII-tubulin-negative patients. Furthermore, no correlation was evident between $TUBB3$, $TACC3$ and $CAPG$ gene expression, MDR1 protein expression, multi-gene expression models, and the efficacy of ixabepilone or paclitaxel, even within the estrogen-receptor-negative subset.

**Conclusion:** These results indicate that βIII-tubulin protein and mRNA expression, MDR1 protein expression, $TACC3$ and $CAPG$ gene expression, and multi-gene expression models (20- and 26-gene) are not predictive markers for differentiating treatment benefit between ixabepilone and paclitaxel in early-stage breast cancer.
**Introduction**

Breast cancer is a heterogeneous disease that can be classified into subgroups on the basis of hormone receptor status, human epidermal growth factor receptor 2 (HER2) expression levels, and gene expression profiling (1–3). Some breast cancer subgroups may have high response rates to specific chemotherapeutic drugs, whereas others may derive a relatively small benefit, but at the same time be exposed to treatment-related toxicity (4–8). This underscores the need for predictive biomarkers that can be used prospectively to select which breast cancer patients are most likely to respond to a given treatment, and which should be offered an alternative regimen with a greater likelihood of benefit. Accordingly, predictive biomarkers offer the potential to improve the benefit:risk ratio of a given therapeutic agent.

The neoadjuvant setting provides an opportunity for identifying biomarkers that are predictive of treatment response in breast cancer patients (9,10). Pathologic complete response (pCR) after neoadjuvant therapy is associated with favorable disease-free survival and overall survival (11–13), and is thus a valid endpoint for correlation with biomarker expression. Indeed, various gene expression profiles have been shown to correlate with pCR after neoadjuvant chemotherapy regimens in breast cancer patients (14–26).

Ixabepilone, an epothilone agent that is currently approved for the treatment of chemotherapy-resistant metastatic breast cancer in the USA, achieves a pCR rate (18% in a single arm phase II study) similar to that seen with other agents commonly used in the neoadjuvant setting (i.e. docetaxel, paclitaxel, and doxorubicin/cyclophosphamide [AC]) (27–31). Ixabepilone has a similar, but distinct, mechanism of action to that of taxanes, and appears to be less sensitive to mechanisms that confer taxane resistance(32,33). Current research is focused on the role of ixabepilone in patients with early relapse after taxane-based adjuvant therapy.
Preclinical evidence and retrospective analyses of clinical studies suggest that expression of βIII-tubulin may be a valid biomarker of differential tumor sensitivity to ixabepilone and the taxanes in breast cancer (34–39). These data indicate that βIII-tubulin confers resistance to paclitaxel, but not to ixabepilone. In-vitro, βIII-tubulin expression correlates with degree of resistance to paclitaxel in breast cancer cell lines (38).

Downregulation of the expression of βIII-tubulin by various manipulations (RNAi, antisense, hypoxia, etc), consistently increases sensitivities of cell lines to paclitaxel, while upregulation of βIII-tubulin expression decreases sensitivity to paclitaxel (35,36). In contrast, ixabepilone retains activity in taxane-resistant tumor cells with high βIII-tubulin expression (33).

In the neoadjuvant setting (27), ixabepilone monotherapy demonstrates a higher pCR rate among estrogen-receptor (ER)-negative patients than in the overall population (29% vs. 18%). Further analysis of this patient subgroup indicates a markedly higher pCR rate in those with ER-negativity and overexpression of βIII-tubulin (n = 24) than patients with ER-negativity, but no βIII-tubulin overexpression (n = 38; 25% vs. 5%) (40). Patients with triple-negative (TN), basal-like or HER2-positive tumors may have higher βIII-tubulin expression than other breast cancer subtypes, which may contribute to the aggressiveness of these subtypes, and predict for ixabepilone clinical response (40).

Studies of gene expression profiles from ER-negative patients who received neoadjuvant ixabepilone or a taxane-containing regimen (neoadjuvant paclitaxel followed by fluorouracil, and AC [FAC]) have identified four other potential biomarkers that differentiate pCR with ixabepilone from the taxane-containing regimen (41). These biomarkers include two microtubule-related genes, transforming acidic coiled-coil-containing protein 3 (TACC3) and chromosome condensation protein G (CAPG), and 20- and 26-gene models. TACC3 localizes to the centriole and has a role in microtubule dynamics (42), and CAPG is a component of a condensin complex that impacts centromere and kinetochore function, although the mechanism is not clearly defined (43).
This randomized, phase II trial was designed to compare pCR rates induced by neoadjuvant AC followed by ixabepilone or paclitaxel in women with early-stage breast cancer, based on the five pre-defined biomarker sets described above: βIII-tubulin protein expression measured by immunohistochemistry (IHC), TACC3 and CAPG gene expression, and expression of 20- and 26-gene lists. This study also compared pCR rates in treatment arms defined by multidrug resistance protein 1 (MDR1) protein expression, and assessed the predictive value of mRNA expression of TUBB3 and other β-tubulin isoform genes; preclinical evidence has suggested that β-tubulin isoforms other than βIII-tubulin may also exhibit resistance to taxanes (44,45).

Methods

Study design and patients

This randomized, open-label, multicenter, phase II trial (NCT00455533) enrolled previously untreated women with histologically-confirmed primary invasive breast adenocarcinoma (T2–3, N0–3, M0, tumor size ≥2.0 cm), regardless of hormone receptor or HER2 expression status. The trial was initially restricted to TN breast cancer (TNBC), but was later amended to include all tumor types, independent of any knowledge of accumulated outcome data, based on slow patient accrual.

Patients received sequential neoadjuvant therapy starting with 4 cycles of AC (doxorubicin 60 mg/m² intravenously and cyclophosphamide 600 mg/m² intravenously) given every 3 weeks, followed by 1:1 randomization to either ixabepilone (40 mg/m² 3-hour infusion) every 3 weeks for 4 cycles, or paclitaxel (80 mg/m² 1-hour infusion) weekly for 12 weeks. Patients were stratified by tumor size at baseline, ER status, clinical response to AC and investigator site. In order to provide 81% power to detect a 14% or greater difference in
pCR rates using a one-sided, $\alpha = 0.05$ level Fisher's exact test, approximately 300 patients (150 per arm) were randomized to either ixabepilone or paclitaxel.

All patients underwent definitive breast surgery 4–6 weeks after the last dose of ixabepilone or paclitaxel, consisting of either a lumpectomy with axillary dissection or modified radical mastectomy. Surgical specimens were evaluated by a staff pathologist at each study site; no central pathology review was performed. The pCR rate was evaluated as the primary endpoint, with pCR defined by no histologic evidence of residual invasive adenocarcinoma in the breast and axillary lymph nodes, with or without the presence of ductal carcinoma in situ. Full details of the study design are reported separately (Saura et al., manuscript in preparation).

The trial was conducted in accordance with the ethical principles originating in the Declaration of Helsinki, and in compliance with Good Clinical Practice and regulatory guidelines. The study was approved by the Institutional Review Board or Independent Ethics Committee at all participating sites. All patients provided written informed consent.

**Tissue specimens**

Four core needle tumor tissue biopsies (4 passes) were obtained before neoadjuvant therapy with AC. Three biopsy specimens were combined at the study site and immediately placed in RNAlater® solution for subsequent gene expression analysis. The remaining biopsy specimen was formalin-fixed and paraffin embedded (FFPE) at the study site, and then underwent immunohistochemical analysis for selected protein antigens.

In a small subset of patients with incomplete pathological information prior to study entry, an additional (fifth) core needle biopsy was obtained (during the same procedure at the study site) to confirm the diagnosis of invasive carcinoma and assess HER2, ER and progesterone-receptor (PR) status.
Analysis of mRNA expression

The mRNA expression levels of 2 single gene models, TACC3 and CAPG, as well as the multi-gene (20- and 26-gene) expression models were measured using an Affymetrix gene expression profiling approach. Affymetrix gene expression data are available from the Gene Expression Omnibus website (GSE41998).

The core tumor biopsy specimens in RNAlater® solution were processed for extraction of total RNA. Downstream labeling reactions were conducted on all RNA samples with an RNA integrity number (RIN) ≥2.8 and >50 ng total RNA. Biotin-labeled cRNA targets were synthesized using an Affymetrix in-vitro transcription (IVT) labeling kit, and all labeled cRNA targets with ≥10 µg yield of product were then hybridized to Affymetrix Human Genome (HG) U133A 2.0 GeneChips. Hybridization quality was assessed using several different metrics, including scaling factor, percentage of probesets above the threshold of detection, and ratios of intensity of 3’ and 5’ probes for two ubiquitously expressed genes. Probesets representing the 2 single-gene models (TACC3 and CAPG), the 20- and 26-gene models, and the β-tubulin genes are present on the HG U133A 2.0 GeneChips. Intensity levels detected by the corresponding probes were normalized using a Robust Multi-Chip Average method. The resultant values correlated with mRNA expression levels for each of the genes. When genes were represented by multiple probes, the average expression level was calculated.

Breast cancer subtyping

Gene expression profiling data was used for molecular subtyping: genes that constitute the PAM50 (46) were converted into Affymetrix probesets; hierarchical clustering using centroid linkage in Array Studio to define basal-like, luminal-like A, luminal-like B, HER2-enriched, and normal-like subtypes.
In addition, information regarding HER2, ER and PR status was collected from the sites that participated in the clinical study. This information was used to classify subjects with triple negative (HER2-negative, ER-negative and PR-negative) or non-triple negative breast cancer.

**βIII-tubulin IHC**

βIII-tubulin protein expression was measured by IHC using a prototype pharmacodiagnostic assay developed by Dako North America, Inc. (Carpinteria, CA). The assay was based on previously reported IHC assays for βIII-tubulin (47,48). βIII-tubulin cytoplasmic staining was scored on a 0–3 scale (negative, weak, moderate, and strong), and the percentage of tumor cells at each intensity level was determined. Endothelial cells present in most tissue specimens, which consistently stained at a 2–3 intensity level, were used as an internal positive control. An isotype matched antibody was used as a negative control to evaluate background staining. A pre-specified cut-off for βIII-tubulin-positive staining was defined as staining in ≥50% of tumor cells at an intensity of 2–3. In addition, the Histo-score of βIII-tubulin staining was determined from the following formula:

\[
\text{Histo-score} = 100 \times (\% \text{ cells with intensity 1}) + 200 \times (\% \text{ cells with intensity 2}) \\
+ 300 \times (\% \text{ cells with intensity 3})
\]

**MDR1 IHC**

MDR1 protein levels were measured by IHC using a protocol adapted from previously published methods and included the use of a monoclonal antibody (49). Briefly, four micron sections were deparaffinized and epitope recovered by the steam heat induced epitope recovery method described by Ladner and colleagues (50). Subsequent to a 15 minute incubation with UltraVision block at room temperature, tissue sections were incubated with
the JSB-1 antibody (dilution 1:150; Santa Cruz Biotechnology) overnight. Antibody binding was detected with the diaminobenzidine-based UltraVision chromogenic detection system. Slides were counterstained with Hematoxylin. The method was optimized to reduce background cytoplasmic staining and enhance membrane staining; however, cytoplasmic staining (presumably cross-reactivity with another protein) was not completely eliminated. Average cytoplasmic and membrane staining intensities were measured on a 0–3 scale (negative, weak, moderate, and strong), and the percentage of tumor cells with any staining was also captured for both cytoplasmic and membrane localizations. Two thresholds were used to define patients with negative and positive MDR1 status with the first threshold stringently defining IHC-positive status as any membrane staining, and the second threshold being more inclusive of samples with any membrane staining or cytoplasmic Histo-score of ≥200.

Statistics

βIII-tubulin, TACC3 and CAPG gene expression, as well as expression of other β-tubulin genes, were also summarized descriptively. For each, a logistic regression model (full model) was built with biomarker expression, treatment status, ER status, and all 2- and 3-way interactions as covariates, and pCR as a response variable. In addition, a reduced model was constructed with treatment status, ER status, and their interaction as covariates, and another reduced model with biomarker expression, treatment status, and their interaction as covariates. Comparison of the full and reduced models was made using a likelihood ratio test.

To identify the optimal threshold level for each biomarker-defined subpopulation, a logistic model was constructed using pCR status as the response variable and biomarker status, treatment status, and their interaction as covariates. The cut-off for biomarker
positivity was identified by the minimal $P$ value of the interaction, with the constraint that the prevalence of the biomarker-defined subpopulation was $>15\%$ and $<85\%$.

A multi-gene expression model was built for the 20- and 26-gene biomarker sets using penalized logistic regression for each treatment arm separately. Receiver operating characteristic (ROC) plots were generated using 5-fold cross-validation within each arm. For each arm, patients were partitioned into 5 equal sized subsets. Four subsets were used as the training set to fit the multi-gene model and the fifth subset was used as the testing set to calculate sensitivities and specificities. All of the 5 subsets were rotated as the testing set and the weighted averages of the sensitivities and specificities were used to generate the ROC plots.

The pCR rate and 90% confidence interval (CI) in the ixabepilone and paclitaxel arms in biomarker population defined by βIII-tubulin protein, TACC3 and CAPG gene expression were estimated by the cross-validation method using a 5-fold cross-validation scheme. The secondary efficacy endpoint, pCR/minimal residual cancer burden (RCB-1) rate, was also analyzed by the cross-validation method using a 5-fold cross-validation scheme. RCB was calculated as a continuous index combining pathologic measurements of primary tumor and nodal metastases for prediction of distant relapse-free survival in multivariate Cox regression analyses. Detailed methodology has previously been reported (51).

**Results**

**Patient disposition and baseline characteristics (biomarker evaluable population)**

A CONSORT diagram detailing the patients that were enrolled, randomized, and assessed for gene expression analysis, βIII-tubulin IHC, MDR1 IHC, and pCR/RCB-1 data is
shown in Figure 1. The baseline characteristics of patients in the biomarker evaluable population and the entire study cohort are described in Supplemental Table 1.

Out of a total of 295 patients randomized in the study, RNAlater® specimens for mRNA expression profiling were submitted for 283 patients; however, specimens from 10 patients yielded poor quality RNA (RIN ≤2.8), and RNA from an additional 13 patients did not meet labeling standards for hybridization to Affymetrix GeneChips (IVT yield <10 µg). Therefore, gene expression data from 260 randomized patients was available; 15 patients in this subset did not have pCR data, thus a total of 245 patients had both gene expression profiling and pCR data available (Supplemental Table 2).

βIII-tubulin IHC data was available for 247 randomized patients (Saura, et al., manuscript in preparation); 16 patients in this randomized subset did not have pCR data, thus a total of 231 patients had both βIII-tubulin IHC data and pCR data available.

FFPE tumor tissue was available from 290 randomized patients for MDR1 IHC assessment; however, IHC data was available from only 244 patients, as the submitted sample sections for 42 patients had no evidence of tumor, and sections for 4 patients had no tissue present. An additional 16 patients did not have available pCR data, so 228 randomized patients had MDR1 IHC data available.

Predictive value of the single- and multi-gene expression models

For single gene models, TACC3 and CAPG, mRNA expression was measured using Affymetrix gene expression chips, and an optimized cut-off of normalized log2 expression was established. However, neither model predicted benefit for ixabepilone versus paclitaxel: pCR rates did not differ between treatment arms, and logistic regression did not identify any significant correlation between TACC3 and CAPG, mRNA expression and treatment.
response (Table 1; Supplemental Table 3). A similar finding was evident in the ER-negative subset (data not shown).

Multi-gene expression models also did not differentially predict pCR between treatment arms (the genes that constitute these multi-gene expression models are shown in Supplemental Table 4). ROC curves generated separately for the ixabepilone and paclitaxel arms using the cross-validation method did not indicate that the 20- and 26-gene models differentially predicted pCR between treatment arms (Fig. 2). Further analyses to estimate the optimal cut-off and pCR rates in positive and negative groups were consequently not conducted in these multiple-gene models.

**Prevalence and predictive value of βIII-tubulin**

Expression of βIII-tubulin was assessed at both the protein level, using an IHC assay, and at the mRNA level, using data from Affymetrix gene expression data (IHC data from this study are reported separately [Saura et al., manuscript in preparation], and also included here for completeness).

A correlation between protein and mRNA levels for βIII-tubulin was observed. Relative βIII-tubulin mRNA expression levels were significantly higher in patients classified as βIII-tubulin-positive by IHC compared with those classified as βIII-tubulin-negative ($P < 0.0001$; Supplemental Fig. 1). In addition, gene expression correlated with βIII-tubulin IHC Histo-score ($r = 0.49$).

Eighty-two percent (108/132) of TN specimens with gene expression data were classified as basal-like; 47% (106/224) of randomized patients with both βIII-tubulin IHC and gene expression data were classified as basal-like by intrinsic gene clustering. Distribution of βIII-tubulin positivity (defined using a pre-specified cut-off) was non-random amongst subtypes (Table 2), with a significantly higher frequency in basal-like and HER2-enriched
versus other breast cancer subtypes \( (P < 1 \times 10^{-5}, \text{Chi-square test}) \). Fifty-five percent (58/106) of basal-like specimens were classified as \( \beta \)-III-tubulin-positive. Conversely, 67% (58/86) of \( \beta \)-III-tubulin-positive specimens were classified as basal-like.

The pCR rate for the overall study population was similar between the treatment arms (ixabepilone: 24.3%; paclitaxel: 25.2%), and similar to that reported historically for anthracycline- and taxane-based regimens in this setting. The pCR rates for the subset of patients with tumor specimens for both gene expression analysis are provided in Table 1, along with pCR rates for the entire study cohort.

Sensitivity analyses were conducted using pCR/RCB-1 as the endpoint; and evaluating pCR in the subset of ER-negative patients; and \( \beta \)-III-tubulin data per mRNA expression (Table 1). The results were consistent, with no correlation observed between biomarker and treatment outcome.

**Predictive value of MDR1**

There was no significant difference in pCR or pCR/RCB-1 between ixabepilone and paclitaxel for MDR1 protein levels (Table 1). pCR rates were also assessed within the ER-negative subset, and again no association between treatment and MDR1 IHC status was observed (data not shown).

**Discussion**

Neoadjuvant chemotherapy is an effective treatment option for patients with operable breast cancer. This type of therapy, in which tumor tissue is collected before and after chemotherapy, allows for biomarker analyses to guide patient selection (predictive biomarker), assess the biologic effect of treatment (pharmacodynamic biomarker), and risk stratification (prognostic biomarker). Identifying a biomarker that differentially predicts benefit
within a class of chemotherapeutic agents, such as microtubule stabilizing agents, could help patients to achieve the maximal clinical benefit from therapy.

Ixabepilone has well-established clinical activity in taxane-resistant breast cancer (52–54). Several candidate biomarkers have been proposed that may predict a differential benefit of ixabepilone versus paclitaxel in breast cancer, based on prior published literature and/or preclinical/clinical evidence (35,36,38,41). These include βIII-tubulin protein and mRNA expression, TACC3 and CAPG gene expression and the multi 20- and 26-gene models. Although this randomized, phase II study was adequately designed to explore the potential for these candidate biomarkers, no correlation was seen between any biomarker and differential treatment response (pCR) to ixabepilone and paclitaxel (both with prior AC).

Clinically, overexpression of βIII-tubulin has been associated with resistance to paclitaxel in many tumor types, including breast, ovarian, and non-small cell lung cancer (37,38,47,48). Given the activity of ixabepilone seen in taxane-resistant tumor cells with high βIII-tubulin expression (33), it was expected that ixabepilone would demonstrate greater efficacy than paclitaxel among βIII-tubulin-positive patients. However, the results of our study did not show βIII-tubulin protein and mRNA expression to be predictive markers for differentiating treatment benefit between ixabepilone and paclitaxel. While higher pCR rates were seen among βIII-tubulin-positive patients compared with βIII-tubulin-negative patients, this was true for both the ixabepilone- and paclitaxel-treated cohorts. This suggests that overexpression of βIII-tubulin may be associated with a general increase in sensitivity to chemotherapy.

Our data (plus that reported by Saura et al. manuscript in preparation) show that βIII-tubulin overexpression correlates with TNBC, a subtype of breast cancer with increased chemo-sensitivity relative to ER-positive breast cancer (7). Fifty-three percent of TN specimens were classified as βIII-tubulin-positive, compared with only 22% (21/97) of ER-positive patients and 28% (7/25) or HER2-positive patients. Ixabepilone has demonstrated
efficacy in TNBC (55). However, long-term follow-up data are required to determine the prognostic value of βIII-tubulin overexpression; the current trial was not designed to collect long-term data.

Previous data indicate that ER-negativity is associated with increased pCR in patients receiving ixabepilone neoadjuvant therapy (27). When comparisons were made between ER-negative patients receiving neoadjuvant ixabepilone and those receiving a paclitaxel-containing regimen (paclitaxel followed by FAC) in another trial, TACC3 and CAPG gene expression, and the 20- and 26-gene expression models were found to differentiate ixabepilone-induced pCR (41). However, the present study showed no apparent correlation between TACC3 and CAPG gene expression, MDR1 protein expression, multi-gene expression models, and the efficacy of ixabepilone or paclitaxel, even within the ER-negative subset (Supplemental Table 3).

AC may have had a confounding effect in the present study. Although there is a lack of evidence suggesting a relationship between AC sensitivity and βIII-tubulin status, an interaction between biomarker expression and AC clinical activity cannot be ruled out. Treatment with AC within the current study may have muted any potential correlation between gene expression models and treatment with a microtubule stabilizing agent. Furthermore, this study may have had additional power if it was carried out within TN or ER/HER2-negative populations only.

The dosing schedule of ixabepilone has been assessed in the first-line treatment of metastatic breast cancer. In a randomized, phase II study, ixabepilone (40 mg/m²) every 3 weeks was more active than weekly ixabepilone (16 mg/m²), in combination with bevacizumab (56). A more recent Cancer and Leukemia Group B (CALGB) phase III study demonstrated that weekly ixabepilone in combination with bevacizumab was not superior to weekly paclitaxel in combination with bevacizumab (57). These results support selection of
every-three-week ixabepilone dosing in the present study, in which pCR rates were similar in both the ixabepilone and paclitaxel arms.

No examples of a validated predictive biomarker for individual chemotherapeutic regimens have yet been described, although numerous gene signatures and candidates (ERCC1, RRM1, etc) have been proposed. The reason for this may be attributed to the complexity and redundancy of pathways related to resistance to cytotoxic agents. Alternatively, the prevalence of biomarkers/signatures that may discriminate between 2 cytotoxic agents, may be so infrequent that they cannot be properly tested in phase II studies. The current phase II study highlights the challenges in development of predictive biomarkers and the need to conduct properly designed, prospective, randomized studies in validating predictive biomarker before launching a phase III study.

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All table and figure legends

**Table 1.** Biomarker evaluable populations: efficacy data

**Table 2.** Association between βIII-tubulin status and breast cancer subtype

**Figure 1.** CONSORT diagram. *Assessments performed on core needle tumor tissue biopsies obtained before neoadjuvant therapy with AC. AC, doxorubicin plus cyclophosphamide; AE, adverse event; IHC, immunohistochemistry; MDR1, multidrug resistance protein 1, pCR, pathologic complete response; RCB-1, residual cancer burden index.

**Figure 2.** Multi-gene expression models: ROC curves. ROC, receiver operating characteristic; AUC, area under the curve.
Table 1. Biomarker evaluable populations: efficacy data

<table>
<thead>
<tr>
<th>Patient group</th>
<th>Ixabepilone</th>
<th>Paclitaxel</th>
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<tbody>
<tr>
<td></td>
<td>n</td>
<td>pCR rate, % (90% CI)</td>
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<tr>
<td>All randomized</td>
<td>148</td>
<td>24.3 (18.6–30.8)</td>
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<tr>
<td>All treated</td>
<td>145</td>
<td>24.8 (19.0–31.4)</td>
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<tr>
<td><strong>TACC3 mRNA-positive</strong></td>
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<td><strong>TACC3 mRNA-negative</strong></td>
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<td>21.1 (10.5–33.4)</td>
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<td><strong>CAPG mRNA-positive</strong></td>
<td>–</td>
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<td><strong>CAPG mRNA-negative</strong></td>
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<td>MDR1 IHC-positivea</td>
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<tr>
<td>MDR1 IHC-negativeb</td>
<td>92</td>
<td>22.8 (15.8–31.2)</td>
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</tbody>
</table>

Abbreviations: pCR, pathologic complete response; CI, confidence interval; TACC3, transforming acidic coiled-coil-containing protein 3; CAPG, chromosome condensation protein G; MDR1, multidrug resistance protein 1; IHC, immunohistochemistry.

aAny MDR1 membrane staining or at least 200 cytoplasmic Histo-score.

bAny MDR1 membrane staining.
Table 2. Association between βIII-tubulin status and breast cancer subtype

<table>
<thead>
<tr>
<th>Tumor classification</th>
<th>βIII-tubulin expression&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive (n = 86)</td>
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<tr>
<td>Intrinsic breast cancer subtypes (N = 224)&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Basal-like (106; 47%)</td>
<td>58 (55)</td>
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<tr>
<td>HER2-enriched (17; 8%)</td>
<td>9 (53)</td>
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<tr>
<td>Luminal-like A (40; 18%)</td>
<td>8 (20)</td>
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<tr>
<td>Luminal-like B (50; 22%)</td>
<td>10 (20)</td>
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<td>Normal-like (11; 5%)</td>
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</table>

Abbreviations: HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry.

<sup>a</sup>Pre-specified cut-off (defined as ≥ 50% 2+ or 3+ cells)

<sup>b</sup>224 patients had both βIII-tubulin IHC data and gene expression profiling data (Saura et al. manuscript in preparation).
Figure 1

- Patients enrolled (N = 384)
  - Patients treated with AC (n = 313)
  - Patients not randomised (n = 18)
    - Disease progression (n = 5)
    - Withdrawn consent (n = 3)
    - No longer met study criteria (n = 2)
    - Toxicity due to AC (n = 2)
    - AE unrelated to AC (n = 1)
    - Lost to follow-up (n = 2)
    - Not reported/other (n = 3)
  - Patients randomised (n = 295)
    - Ixabepilone (n = 148)
      - Not continuing to surgery (n = 6)
        - Study drug toxicity (n = 2)
        - Disease progression (n = 1)
        - Withdrawn consent (n = 2)
        - Other (n = 1)
      - Underwent surgery (n = 139)
        - Assessed for βIII–tubulin IHC* (n = 123)
        - Assessed for mRNA expression* (n = 137)
        - Assessed for MDR1 IHC* (n = 120)
        - Assessed for βIII–tubulin IHC* and pCR/RCBI (n = 114)
    - Paclitaxel (n = 147)
      - Not continuing to surgery (n = 6)
        - Death (n = 3)
        - Disease progression (n = 1)
        - Withdrawn consent (n = 1)
        - Lost to follow-up (n = 1)
      - Underwent surgery (n = 138)
        - Assessed for βIII–tubulin IHC* (n = 124)
        - Assessed for mRNA expression* (n = 123)
        - Assessed for MDR1 IHC* (n = 124)
        - Assessed for βIII–tubulin IHC* and pCR/RCBI (n = 117)
Biomarker Analysis of Neoadjuvant Doxorubicin/Cyclophosphamide Followed by Ixabepilone or Paclitaxel in Early-stage Breast Cancer

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