*Advances in Brief*

# Paclitaxel-induced Apoptosis and Mitotic Arrest Assessed by Serial Fine-Needle Aspiration: Implications for Early Prediction of Breast Cancer Response to Neoadjuvant Treatment[1]

**W. Fraser Symmans,[2] Matthew D. Volm, Richard L. Shapiro, A. Bridget Perkins, Alice Y. Kim, Sandra Demaria, Herman T. Yee, Heather McMullen, Ruth Oratz, Paula Klein, Silvia C. Formenti, and Franco Muggia**

Departments of Pathology [W. F. S., S. D., H. T. Y.], Medicine [M. D. V., R. O., P. K., F. M.], Surgery [R. L. S., H. M.], Radiology [A.Y. K.], and Radiation Oncology [S. C. F.], and Kaplan Comprehensive Cancer Center, New York University Medical Center, New York, New York 10016

## Abstract

**The extent of tumor reduction from neoadjuvant chemotherapy for breast cancer correlates with outcome. We investigated whether the initial cellular responses to paclitaxel are related to the extent of tumor reduction. Eleven women with breast cancer received paclitaxel (every 2 weeks for 4 cycles) as neoadjuvant treatment. Serial fine-needle aspirations (FNA; 25-gauge, 1 pass) were obtained before treatment and at 24, 48, 72, and 96 h after the first paclitaxel dose. Microscopic counts of apoptotic and mitotic indices were performed. The change in cancer volume from treatment was determined using radiological measurements with allowance for change in the histopathological amount of cancer. Apoptotic and mitotic responses usually subsided within 4 days. The duration of the initial apoptotic response was different for women with different treatment results. Cumulative apoptotic response for the first 4 days inversely correlated with the proportion of residual cancer after neoadjuvant treatment. FNA is a versatile clinical method to obtain breast cancer cells for therapy response studies. Apoptotic response to the first dose of paclitaxel is almost complete within 4 days, implying that more frequent (weekly) paclitaxel dosing might be beneficial. The apoptotic response to the first dose of paclitaxel appeared to predict the amount of cancer reduction from this treatment. This is a promising start toward the development of an early chemopredictive assay for paclitaxel treatment of breast cancer.**

## Introduction

FNA[3] has potential scientific and clinical applications to the treatment of cancer that go beyond its established role as a diagnostic technique. FNA is a quick, minimally invasive procedure in experienced hands, is well tolerated by patients, and can deliver within seconds an almost pure sample of cancer cells directly from the patient's tumor into whichever media or fixative is required for specific assays. Therefore, FNA is a potentially useful technique to study *in vivo* the cellular changes that occur during the course of cancer treatment. We used serial FNAs to examine cytological responses in breast cancers during the 96 h following the first dose in a preoperative (neoadjuvant) paclitaxel chemotherapy regimen.

The two main reasons supporting the treatment of breast cancer with neoadjuvant (rather than conventional postoperative) chemotherapy are: (*a*) to predict patient outcome based on the observed response to treatment; and (*b*) to reduce the size of the primary tumor and thus facilitate surgical management (1–4). Clinical trials have indicated that the extent of response of the primary tumor to neoadjuvant chemotherapy correlates with disease-free and overall survival (1, 2, 5–7). On the basis of the clinicopathological status of patients at the time of surgery (after neoadjuvant chemotherapy; Ref. 8), a minority (3–12%) of patients obtain complete pathological response, but these patients have the longest survival (1, 2, 5, 7). Another minority of patients (12–25%) who obtained minimal or no pathological response (<50% tumor diameter reduction by clinical measurement) fare significantly worse, whereas the majority (70–80%) of women with a partial response to treatment have an intermediate outcome (1, 2). Indeed, the outcomes for women with a partial response to treatment are probably variable, and these differences may be difficult to predict based solely on clinicopathological response. If the extent of tumor reduction at the completion of neoadjuvant treatment predicts for patient survival, then early prediction of tumor response to the neoadjuvant chemotherapy could yield useful information. Early identification of women who are expected to have no response or an incomplete response could enable their selection as candidates for modification or intensification of their neoadjuvant treatment, possibly including the selection of specific additional agents to augment the efficacy of cancer cell killing (4, 9, 10).

Received 5/18/00; revised 10/3/00; accepted 10/13/00.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] Supported by National Cancer Institute Grant R21 CA66229 pilot study funds (to W. F. S.), a New York State Health Science Research Board Department of Health EMPIRE award (to W. F. S.), an American Cancer Society grant (to M. D. V.), and a California Breast Cancer Research Program grant (to S. C. F.). Presented in part at the American Association for Cancer Research special meeting "Molecular Determinants of Sensitivity to Anti-Tumor Agents," Canada, March 1999.
[2] To whom requests for reprints should be addressed, at Department of Pathology, Box 85, University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: (713) 792-7962; Fax: (713) 745-8610; E-mail: fsymmans@mdanderson.org.

[3] The abbreviations used are: FNA, fine-needle aspiration; HCF, histopathological correlation factor.

An important challenge is to accurately and noninvasively assess the extent of cancer that is present before and after treatment, particularly if tumor response is to be used as an end point for clinical studies. Of note, approximately 1 of 4 women who have a clinical complete response (no palpable tumor) actually have a pathological complete response (3). Conventional breast imaging offers an accessible and promising approach. Studies of breast cancers, with or without neoadjuvant chemotherapy, have also shown that ultrasound measurements predict pathological tumor size more accurately than mammographic or clinical measurements (11–13), although this conclusion is not unanimous (14). Ultrasound measurements in three planes allow for approximation of tumor volume before and after treatment, and therefore a change in volume can be determined as an objective parameter of response. The advantage of this approach is quantification of tumor response that can be compared with potential predictors of outcome.

Paclitaxel is a taxane with activity against breast cancer. *In vitro* treatment of cells with paclitaxel rapidly induces the accumulation of cells in $G_2$-M phases of the cell cycle (after polymerization of microtubules) and leads to apoptosis in susceptible cells (15–17). In paclitaxel-treated MCF-7 breast cancer cells, apoptotic bodies are seen by light microscopy within 24 h of treatment and peak at 48–72 h (18, 19). In a mouse breast cancer model (an inoculated transplantable, spontaneous, mammary carcinoma in C3Hf/Kam mice), accumulation of mitotic figures is seen with light microscopy at 9 h, and apoptotic indices peak at 18–24 h after a single dose (60 mg/kg) of paclitaxel (20). Both mitotic and apoptotic microscopic indices return to pretreatment levels by 4 days in this model (20). The peak apoptotic index and the pretreatment apoptotic index correlated with murine tumor reduction by paclitaxel therapy (21). These preclinical data support our hypothesis that early cellular responses after the first dose of neoadjuvant paclitaxel chemotherapy for breast cancer predict for the extent of tumor reduction.

## Patients and Methods

Women with primary breast cancer were offered a neoadjuvant paclitaxel protocol if the breast tumor had a greatest diameter of at least 2 cm and there was no evidence of systemic metastatic disease. Institutional review board consent was separately obtained for treatment and for serial FNAs from those who selected this therapy. Four pretreatment, 14-gauge core biopsies of the tumor were obtained from different parts of the tumor mass and submitted in 10% neutral buffered formalin solution for routine histopathological analysis. The neoadjuvant paclitaxel chemotherapy dose (200 mg/m$^2$) was administered over 3 h as an i.v. infusion and was given every 2 weeks for a total of four cycles. Postoperative chemotherapy (doxorubicin and cyclophosphamide) and radiation therapy were administered as adjuvant treatment. Adjuvant tamoxifen was added for those patients whose tumor expressed estrogen receptors.

Women who opted for neoadjuvant paclitaxel therapy were also invited to have serial FNAs to assess cellular responses to the first dose of paclitaxel. In this protocol, a baseline FNA was performed before the core biopsy (prior to chemotherapy) and at 24, 48, 72, and 96 h after the first paclitaxel infusion began. All samples were from the primary breast tumor, not from palpable lymph nodes. At each time point, a single-pass FNA was performed using a 25-gauge needle, and the cellular sample was divided onto seven glass slides using a spreader slide. The spreader slide was stained with Diff-quik (Allegiance, McGaw Park, IL) for an immediate microscopic interpretation of the specimen adequacy. The first glass slide was immediately fixed in 95% ethanol and then stained with H&E. H&E stain, rather than *in situ* terminal transferase UTP nick end labeling, was used to identify apoptotic cells because there is excellent cytological detail, close inter-observer consistency, and less artifact and loss of specificity using H&E stain (22–24). We abandoned the terminal transferase UTP nick end labeling (TUNEL) assay to identify apoptotic cells in our samples because the majority of cells had positive staining (even in nontreated control samples), possibly because of DNA stand breaks from smearing, drying, and/or formalin fixation (23, 24). Microscopic counts of the number of identifiable mitotic figures or apoptotic bodies in one thousand cancer cells (×400, ×600; Olympus BH2 microscope) were recorded as percentages (indices). The index at each time point was then divided by the pretreatment baseline index (0 h) and expressed as a ratio. The ratio indicates the proportion of change of the index compared with the baseline index and corrects for variability in baseline indices from different patients' tumors. The sum of these relative changes in apoptotic or mitotic index (each compared with baseline) was calculated to assess the cumulative response for the first 4 days of treatment.

Radiological and clinical measurements of the primary tumor were performed prior to the first dose of paclitaxel and after the fourth cycle of paclitaxel (prior to surgery). Clinical measurements were made of the maximal palpable tumor diameter. Mammographic and ultrasound measurements of the tumor dimensions were made in three dimensions [antero-posterior (*AP*), transverse (*T*), and sagittal (*S*)]. The tumor volume was calculated as $\pi/6 \times (AP \times T \times S)$ (Ref. 25). A radiologist reviewed the mammograms and ultrasound images from each patient and independently decided which modality most accurately demonstrated the tumor mass for measurements of both the pre- and posttreatment tumor volumes. After surgery, the pathological specimen was thoroughly sampled and photo-

*Table 1* Heterogeneity of apoptotic and mitotic indices in breast cancer

Heterogeneity of apoptotic and mitotic indices in 7 untreated, resected breast cancers that ranged from 1.5 to 5.0 cm in pathological diameter. CV is the coefficient of variation (SD/mean). The average (mean) CVs were 0.25 for apoptotic index and 0.46 for mitotic index.

| Tumor | No. of sites | Apoptotic index | | Mitotic index | |
|---|---|---|---|---|---|
| | | Mean (%) | CV | Mean (%) | CV |
| 1 | 10 | 11.6 | 0.15 | 1.0 | 0.32 |
| 2 | 10 | 0.7 | 0.39 | 0.2 | 0.63 |
| 3 | 10 | 1.4 | 0.24 | 0.1 | 0.50 |
| 4 | 10 | 1.4 | 0.26 | 0.2 | 0.57 |
| 5 | 10 | 1.2 | 0.24 | 0.3 | 0.58 |
| 6 | 10 | 3.1 | 0.18 | 1.4 | 0.16 |
| 7 | 9 | 1.2 | 0.28 | 0.2 | 0.38 |
| Mean | | 2.9 | 0.25 | 0.5 | 0.46 |

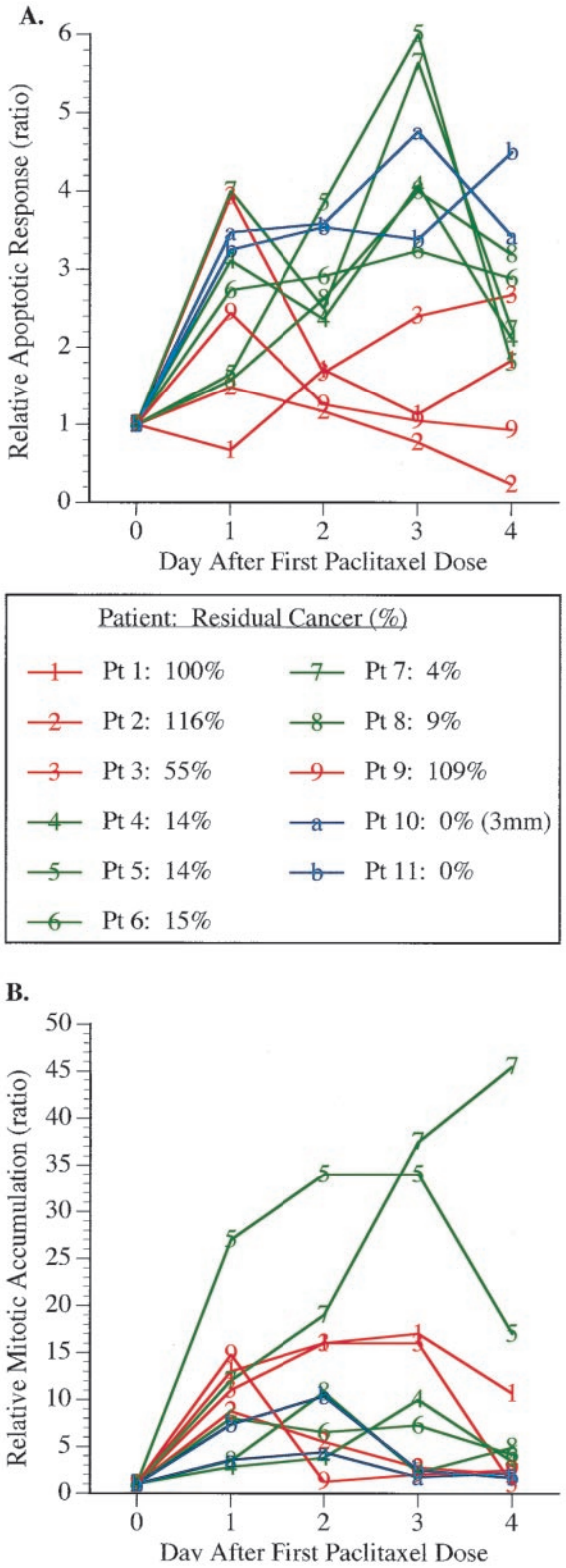


*Fig. 1* Relative changes in indices compared with their pretreatment value are plotted for each day after the first paclitaxel dose for apoptosis (*A*) and mitotic accumulation (*B*). Each patient (*Pt*) is described in the legend according to the tumor response as a percentage of residual cancer volume after treatment (Table 2). Color coding is by the extent of cancer volume reduction.

graphed by the investigators, with measurement and extensive histological sampling of the tumor bed. Preoperative clinical, mammography, and ultrasound measures of greatest residual tumor diameter were compared with the greatest pathological tumor diameter using Pearson's correlation coefficient ($r$) to measure their linear relationship.

We introduced a HCF to approximate the microscopic proportion (0–1) of carcinoma cells within recognizable tumor in the histological sections from the pretreatment core biopsy and the posttreatment tumor bed. This HCF corrects for extensive areas of fibrosis or necrosis that would be identified as tumor using radiological studies. The histologically corrected radiological tumor volumes pre- and posttreatment were called "radiological cancer volume." Each cancer volume was calculated by multiplying the radiological tumor volume with the respective HCF. The proportion of residual cancer after treatment was then calculated as a ratio: radiological cancer volume after treatment divided by radiological cancer volume before treatment.

Quantification of cancer response offers a potential advantage for comparison of response with potential predictors of outcome in small clinical study populations. The same radiological modality was used to measure the tumor volume before and after treatment; therefore, inherent errors of the radiological measurement should be similar and may therefore cancel out when the before and after treatment measurements are expressed as a ratio. We believe that this approach is better than a comparison of two measurements that were derived using different methods, *e.g.*, clinical pretreatment and pathological posttreatment measurements. We compared the observed cellular responses ($G_2$-M arrest and apoptosis) with the proportion of residual cancer after treatment using Pearson's correlation coefficient ($r$). The scatter plots from these pilot data indicated a relationship between cellular response and tumor reduction. We assumed a linear relationship only for simplicity, recognizing that the number of patients in this pilot study are too few for a sophisticated statistical analysis of variables.

A Macintosh G3 computer (Apple Computer, Cupertino, CA) was used with Deltagraph Professional v2.0.3 (SPSS, Inc., Chicago, IL) and Statview v5 (SAS Institute, Inc., Cary, NC) software to produce time response curves, scatter plots, and statistical analyses. Regional heterogeneity of apoptotic and mitotic indices within a tumor mass was evaluated in a separate control group of untreated resected breast cancers. Samples were obtained from up to 10 different sites within the primary tumor using the FNA and sample preparation methods described above. The coefficient of variation (SD/mean) was then calculated for apoptotic index and mitotic index. Ideally, we would also have evaluated the temporal heterogeneity of our measured indices from serial daily FNAs of the tumors in a control group of untreated women, but we believed that was not clinically reasonable. Temporal heterogeneity could be studied in an appropriate animal model (20, 21).

## Results

Fifteen of 22 (68%) subjects who were treated with neoadjuvant paclitaxel chemotherapy (December 1997–October 1999) elected to have at least one FNA after the first paclitaxel

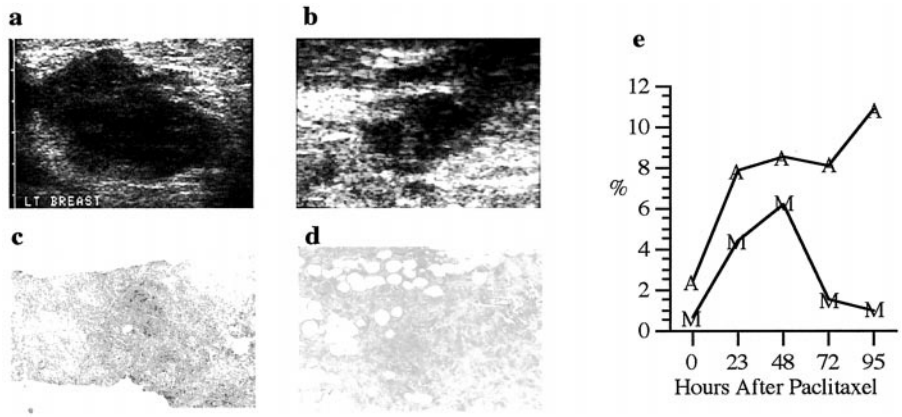


*Fig. 2* Patient 11 (se also Table 2) had a complete pathological response, and this case demonstrates the importance of histological characterization of tumor measured by radiology. The pretreatment ultrasound (*a*) and the posttreatment ultrasound (*b*) were compared and showed a residual hypoechoic 2-cm tumor that was also present on mammograms as a density (not shown). The pretreatment core biopsy showed ~30% cancer by area in the samples (*c*, ×40). At mastectomy, the tumor bed was purely fibrous tissue on histological sections (*d*, ×40). The apoptotic (*A*) and mitotic (*M*) indices are plotted (*e*). There was a sustained apoptotic response and a transient mitotic response.

dose. Twelve patients (55%) had serial FNAs at all five time points, of whom 11 completed the neoadjuvant therapy. The regional heterogeneity of apoptotic and mitotic indices in a separate control group of seven, untreated, resected invasive breast cancers (Table 1) showed average coefficients of variation of 0.25 for apoptotic index and 0.46 for mitotic index. For most of the 11 treated women, there was a 3.0–6.0-fold increase in apoptotic activity (relative to the pretreatment baseline measurement) at some time during the first 4 days after the first dose of neoadjuvant paclitaxel treatment (Fig. 1*a*). There was also a 5.0–50.0-fold relative increase in mitotic index (relative to the pretreatment baseline measurement) at some time during the first 4 days after the first dose of neoadjuvant paclitaxel treatment (Fig. 1*b*). Therefore, the apoptotic and mitotic arrest responses were clearly in excess of the expected range from regional heterogeneity within the tumor.

The apoptotic and mitotic arrest response curves were different for each patient but generally showed an apoptotic response over the first 4 days that was complete or ending by day 4 (Fig. 1*a*). Noticeably, the apoptotic response was sustained for 2 patients (nos. 10 and 11), who had a complete pathological response or a single microscopic residual tumor focus (Figs. 1*a* and 2; Table 2). An apoptotic response either did not occur, or was not sustained beyond day 1, in 3 patients whose cancer volume did not decrease after treatment (patients 1, 2, and 9; Fig. 1*a*; Table 2). The profiles of mitotic response were specific for each patient but did not appear to relate to the measured tumor response to treatment (Fig. 1*b*).

Correlation of the pathological greatest tumor diameter at resection was closer to the radiological measurements after neoadjuvant treatment ($r = 0.80$ for ultrasound; $r = 0.78$ for mammography) than to clinical measurements ($r = 0.36$ for clinical palpation; Fig. 3). Therefore, radiological measurements were used for the subsequent analysis of tumor volume response to treatment. A radiologist independently selected ultrasound in 9 patients and mammography in 2 patients as the better imaging technique from which to accurately measure the tumor volume. The clinicopathological status of response to neoadjuvant treatment can be compared with these radiological measurements of tumor volume change in Table 2. These data suggest that the clinicopathological status assessment may sometimes overestimate the extent of tumor reduction from neoadjuvant chemotherapy (patient 9, Table 2; Fig. 4). Radiological volume measurement alone would have underestimated a complete pathological response for patient 11 (Fig. 2; Table 2). As described in "Patients and Methods," we introduced a HCF to rectify any problem of areas of mass not containing cancer. Sometimes, there was more extensive fibrosis after treatment, as demonstrated by differing HCF values in posttreatment specimens from patients 4, 5, 6, 7, and 11 (Table 2). Occasionally, there was a greater proportion of the mass that contained carcinoma after treatment (patient 2). A HCF was not obtainable after paclitaxel therapy for patient 3, because she continued with Adriamycin-based chemotherapy before surgery (Table 2).

The pretreatment baseline apoptotic ($r = 0.44$) and mitotic ($r = -0.39$) indices did not correlate with the proportion of residual cancer after treatment. There appeared to be a correlation between the apoptotic response to the first dose of paclitaxel and the proportion of residual cancer volume after the neoadjuvant treatment (Fig. 1*a*). The relative change in apoptotic index (compared with the pretreatment index) at each day after the first dose did inversely correlate with the proportion of residual cancer volume [$r = -0.51$ (day 1), $-0.86$ (day 2), $-0.88$ (day 3), and $-0.78$ (day 4)] (Fig. 5). The cumulative apoptotic response (sum of relative changes in apoptotic index for days 1–4 after the first dose) showed an even stronger inverse correlation ($r = -0.97$) with the proportion of residual cancer volume (Fig. 6). There was no correlation between the cumulative mitotic response and the proportion of residual cancer after the neoadjuvant therapy ($r = -0.18$) or the cumulative apoptotic response ($r = 0.25$). We note that our sample size (11 patients) is too small for detailed statistical analysis. Linear

*Table 2* Summary: Breast cancer responses

Clinical, radiological, and pathological measurements of greatest tumor diameter in cm. Histopathological tumor type at diagnosis and axillary nodal status at dissection are presented (0, all axillary lymph nodes were negative). HCFs as percentage of cancer area within the tumor sections are shown to compare the calculated percentage of residual tumor volume (radiology alone) and cancer volume (radiology with histology). Cumulative apoptotic and mitotic cellular response values are the sum of the change in index for all time points (days 1 to 4), relative to day 0.

| | Patient nos. | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
| Diameters (cms) (Post:Pre)[a] | | | | | | | | | | | |
| Clinical | 4.5:6.0 | 5.0:5.0 | 5.0:6.0 | 5.0:9.0 | 2.0:4.0 | 2.0:4.0 | 0:2.5 | 0:15.0 | 0:7.5 | 0:6.0 | 0:5.0 |
| Mammography | 4.5:4.7 | 4.5:4.3 | 3.0:3.5 | 3.0:NA | 1.7:2.4 | 3.6:4.5 | 1.4:2.7 | 4.5:8.0 | 3.0:3.7 | 0:2.5 | 2.3:3.7 |
| Ultrasound | 3.4:2.8 | 3.4:3.2 | 2.1:2.7 | 2.7:4.3 | 1.1:2.0 | 1.2:2.2 | 1.2:2.3 | NA | 3.2:2.5 | 0:2.2 | 2.2:2.9 |
| Pathology | 3.2 | 3.7 | NA[b] | 4.5 | 1.8 | 2.0 | 1.2 | 6.2[c] | 2.0 | 0.2 | 0 |
| Histological type | IDC | IDCM | IDC | IDC | IDC | IDC | IDC | IDC | IDC | IDC | IDC |
| Axillary LNs (+) | 7/17 | 0 | NA[b] | 1/15[d] | 2/29 | 0 | 0 | 0 | 0 | 0 | 0 |
| HCF% (Post:Pre) | 30:30 | 90:70 | NA | 30:50 | 50:70 | 10:30 | 30:70 | 50:50 | 30:30 | 30:30 | 0:30 |
| Clin-Path outcome[e] | SD | SD | SD | PR | PR | PR | PR | PR | PR | MiRD | PCR |
| Residual volume | | | | | | | | | | | |
| Tumor (%) | 100 | 90 | 55 | 24 | 20 | 45 | 9 | 9 | 109 | 0 | 20 |
| Cancer (%) | 100 | 116 | 55 | 14 | 14 | 15 | 4 | 9 | 109 | 0 | 0 |
| Cell responses | | | | | | | | | | | |
| Apoptotic | 5 | 4 | 11 | 13 | 12 | 12 | 14 | 11 | 6 | 15 | 15 |
| Mitotic | 57 | 19 | 44 | 112 | 20 | 26 | 114 | 21 | 21 | 12 | 22 |

[a] Post:Pre, posttreatment:pretreatment; LNs, lymph nodes; IDC, invasive ductal carcinoma; IDCM, IDC with medullary features.
[b] Pathologic findings were not available because this patient deferred surgery until after she had completed another course of Adriamycin-containing chemotherapy.
[c] Aggregate diameter measurement from 18 microscopic foci of cancer.
[d] One micrometastasis only.
[e] Clinico-pathologic (Clin-Path) outcome is the assessment of the tumor response as: SD, stable disease (<50% diameter reduction); PR, partial response (≥50% diameter reduction); MiRD, microscopic pathologic residual disease as a single focus (3 mm); and PCR, pathologic complete response (no histologic cancer at resection).

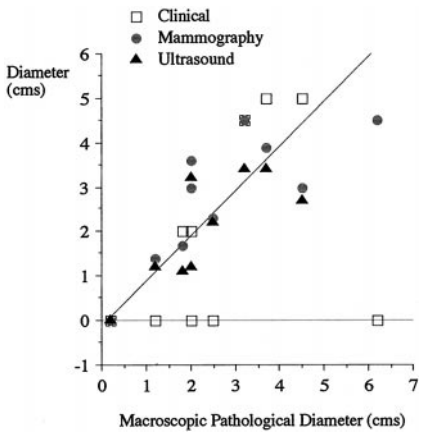


*Fig. 3* The clinical, mammographic, and ultrasound measurements of greatest tumor diameter after neoadjuvant treatment (*Y axis*) are plotted against the pathological measurement of greatest macroscopic tumor diameter at resection (*X axis*). The line with gradient 1.0 represents the pathological diameter (plotted in both axes) as a visual reference of pathological size for each tumor.

correlation coefficients were used to simply describe our observations from the pilot data.

## Discussion

These results show that initial cellular responses to paclitaxel did vary in patients, and that the apoptotic response during the first 4 days after the initial dose of paclitaxel is likely to be a predictive biomarker for the extent of cancer volume reduction. Early prediction of the likely response to treatment could facilitate early modifications to improve an individual's neoadjuvant therapy.

Tumor imaging with measurements in three dimensions, combined with correction for the histopathological proportion of malignant cells within the mass, enabled us to quantify the primary cancer response to neoadjuvant treatment. We observed good correlations between the radiological measurements of greatest tumor diameter immediately prior to surgery and the macroscopic pathological diameter of the tumor bed at resection, which are consistent with most published studies of untreated resected breast cancers (11–13). We note that a quantitative measure of tumor reduction could enable more precise measurement of the treatment response in the majority (70–80%) of patients who have an intermediate (partial) response to neoadjuvant therapy, rather than grouping all of them together (1, 2). Furthermore, if meaningful and predictive information about the early cellular responses to treatment can be obtained using FNA, it could benefit those patients who will have an incomplete (partial) response. Early recognition of such patients might enable a specific early intervention during their treatment to increase the cell killing (by targeting the likely mechanism of impaired chemotherapeutic efficacy) and so improve the benefit from neoadjuvant treatment.

Our limited data did not identify a specific day from which tumor samples should ideally be obtained, because there was variable timing of the response profiles of apoptosis and mitotic accumulation (Figs. 1 and 5). Therefore, we could not accurately

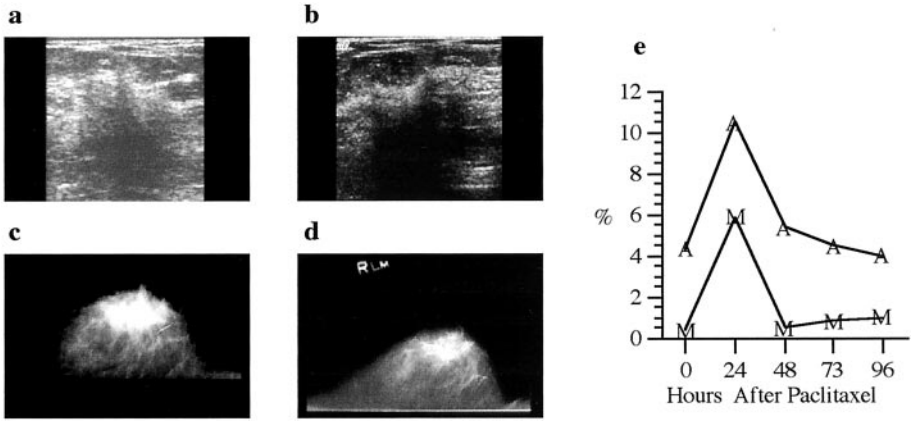


*Fig. 4* Patient 9 (see also Table 2) had a partial response by clinico-pathological assessment, but ultrasound tumor measurements before (*a*) and after (*b*) treatment showed no response (see also Table 2). The pretreatment mammogram (*c*), compared with the posttreatment mammogram (*d*), shows that the density became less opaque, but dimensions were more difficult to measure (than by ultrasound) in this spiculated lesion. There was an initial mitotic and apoptotic response on day 1, but these were abrogated (*e*). The apoptotic (*A*) and mitotic (*M*) indices are plotted.

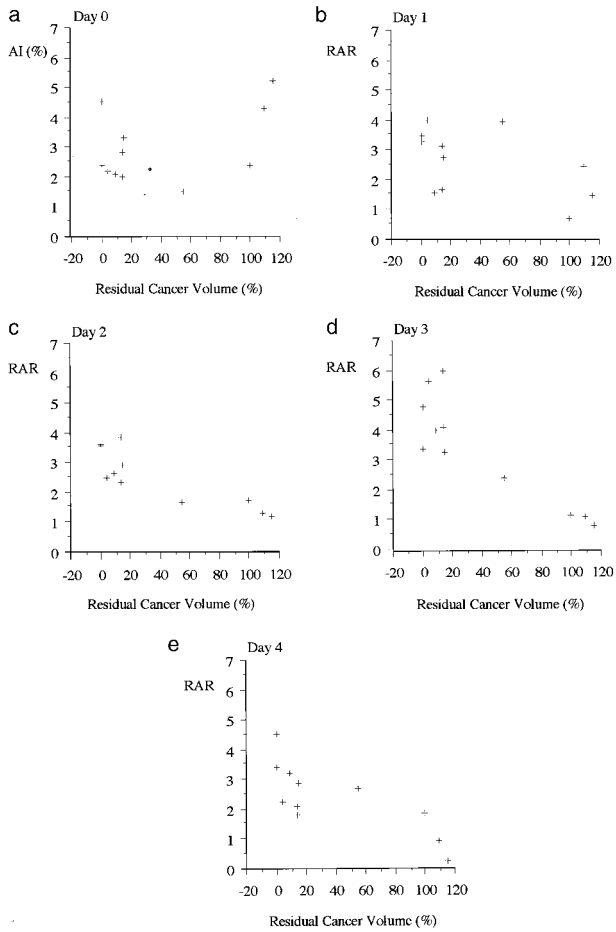


*Fig. 5* Pretreatment (baseline) apoptotic index (*AI*) plotted against the proportion of residual cancer volume after neoadjuvant paclitaxel (*a*). The relative apoptotic response (*RAR*) is the AI from that day compared with the baseline AI for that tumor. RARs for days 1–4 are plotted against the proportion of residual cancer volume (*b–e*).

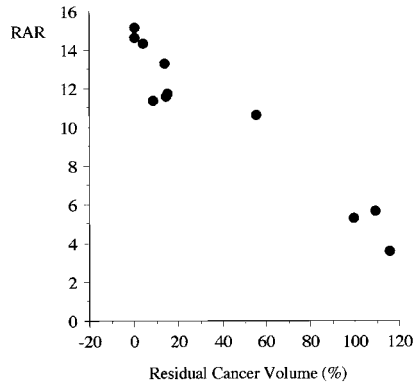


*Fig. 6* Cumulative apoptotic responses for each patient were calculated as the sum of the relative apoptotic response (*RAR*; ratio over baseline value) for days 1–4 after the first dose of paclitaxel. These are plotted against the proportion of residual cancer volume after neoadjuvant paclitaxel.

predict the time to capture cells at the exact peak of cellular response. However, our results suggest that a sustained apoptotic response to treatment is important for greater cancer volume reduction. Further studies might better define the optimal number and timing of samples. Our data from patients, and other published data from mice, show that the apoptotic response to a single dose of paclitaxel lasts for ~4 days (20, 21). This theoretically favors a weekly schedule for neoadjuvant paclitaxel, rather than less frequent dosing intervals of 2–3 weeks. A confirmatory study could be undertaken during a weekly neoadjuvant paclitaxel regimen to assess whether the lower paclitaxel dose in a weekly regimen induces similar apoptotic responses.

The cumulative apoptotic response during the first 96 h after the first dose of paclitaxel had an almost linear relationship to the extent of tumor reduction (Fig. 4). This was despite the observed variability in timing of the apoptotic response in individual patients. Perhaps different waves of apoptosis occur during the first 4 days as different molecular pathways are activated and lead to activation of caspases and apoptosis at different rates (26, 27). For example, one measure of short-term cell death (viability at 24 h) after chemotherapy *in vitro* does not always predict a longer-term anticancer effect (colony formation at 8 days; Ref. 28). The cumulative apoptotic response (from

daily measurements) may correct for the variability in rates of induction of apoptosis by different mechanisms.

It is intriguing to consider whether FNA could be used to study the role of drug resistance mechanisms, and/or inhibition of apoptosis, in breast cancers treated with neoadjuvant chemotherapy. Although the potential mechanisms of resistance to chemotherapy-induced cell killing are myriad (4, 28), the induction of a multiple drug resistance gene (*MDR-1*) product has been implicated as a cause of resistance to neoadjuvant chemotherapy for breast cancer (29–32). Also, blunted and truncated apoptotic responses to neoadjuvant paclitaxel in nonresponsive breast cancers may result from an inability of the cancer cells to efficiently enter and complete the apoptotic pathways that should be induced by paclitaxel (4, 27, 33–38). There is also evidence that extensive apoptosis from taxanes can decrease the intravascular and interstitial pressure in solid tumors, and that may improve drug delivery in subsequent chemotherapy cycles, hence compounding the effectiveness of treatment (39). Knowledge of likely mechanisms of resistance to chemotherapy-induced apoptosis for an individual patient should provide an opportunity for early and targeted intervention to enhance the extent of cell killing, tumor reduction, and survival benefit (9, 10, 40–47).

Larger clinical trials (with statistical power) and more sophisticated molecular analyses of the responses of cancer cells are needed to validate these pilot data. The similarities between our results from patients in a clinical trial and the published preclinical data provide a foundation for more detailed studies of the molecular and cellular responses to paclitaxel chemotherapy in murine models and clinically (20, 21). We have demonstrated in this study that serial FNAs are a minimally invasive adjunct to therapeutic clinical trials with a real potential to obtain samples that can improve our knowledge of breast cancer cellular responses to chemotherapy *in vivo*. This may ultimately allow us to individually tailor each woman's neoadjuvant treatment protocol.

## Acknowledgments

We acknowledge the nursing and administrative support from the General Clinical Research Center, New York University Medical Center, New York, NY, and the commitment of the patients who participated in this study.

## References

1. Hortobagyi, G. N., Ames, F. C., Buzdar, A. U., Kau, S. W., McNeese, M. D., Paulus, D., Hug, V., Holmes, F. A., Romsdahl, M. M., Fraschini, G., McBride, C. M., Martin, R. G., and Montague, E. Management of stage III breast cancer with primary chemotherapy, surgery, and radiation therapy. Cancer (Phila.), *62:* 2507–2516, 1988.

2. Bonadonna, G., Valagussa, P., Brambilla, C., Ferrarri, L., Moliterni, A., Terenziani, M., and Zambetti, M. Primary chemotherapy in operable breast cancer: eight year experience at the Milan Cancer Institute. J. Clin. Oncol., *16:* 93–100, 1998.

3. Fisher, B., Brown, A., Mamounas, E., Wieand, S., Robidoux, A., Margolese, R. G., Cruz, A. B., Fisher, E. R., Wickerham, D. L., Wolmark, M., DeCillis, A., Hoehn, J. L., Lees, A. W., and Dimitrov, N. V. Effect of preoperative chemotherapy on local-regional disease in women with operable breast cancer: findings from National Surgical Adjuvant Breast and Bowel Project B-18. J. Clin. Oncol., *15:* 2483–2493, 1997.

4. Bhalla, K., and Harris, W. B. Molecular and biologic determinants of neoadjuvant chemotherapy of locoregional breast cancer. Semin. Oncol., *25:* 19–24, 1998.

5. Machiavelli, M. R., Romero, A. O., Pérez, J. E., Lacava, J. A., Domínguez, M. E., Rodríguez, R., Barbieri, M. R., Romero Acuña, L. A., Romero Acuña, J. M., Langhi, M. J., Amato, S., Ortiz, E. H., Vallejo, C. T., and Leone, B. A. Prognostic significance of pathological response of primary tumor and metastatic axillary lymph nodes after neoadjuvant chemotherapy for locally advanced breast carcinoma. Cancer J. Sci Am., *4:* 125–131, 1998.

6. Ferrière, J. P., Assier, I., Curé, H., Charrier, S., Kwiatkowski, F., Achard, J. L., Dauplat, J., and Chollet, P. Primary chemotherapy in breast cancer: correlation between tumor response and patient outcome. Am. J. Clin. Oncol., *21:* 117–120, 1998.

7. Kuerer, H. M., Newman, L. A., Smith, T. L., Ames, F. C., Hunt, K. K., Dhingra, K., Theriault, R. L., Singh, G., Binkley, S. M., Sneige, N., Buchholz, T. A., Ross, M. I., McNeese, M. D., Buzdar, A. U., Hortobagyi, G. N., and Singletary, S. E. Clinical course of breast cancer patients with complete pathologic primary tumor and axillary lymph node response to doxorubicin-based neoadjuvant chemotherapy. J. Clin. Oncol., *17:* 460–469, 1999.

8. Hayward, J. L., Carbone, P. P., Heuson, J-C., Kumaoka, S., Segaloff, A., and Rubens, R. D. Assessment of response to therapy in advanced breast cancer. Cancer (Phila.), *39:* 1289–1294, 1997.

9. Luykx-de Bakker, S. A., Verheul, H. M. W., de Gruijl, T. D., and Pinedo, H. M. Prolonged neoadjuvant treatment in locally advanced breast tumours: a novel concept based on biological considerations. Ann. Oncol., *10:* 155–160, 1999.

10. Sarkadi, B., and Müller, M. Search for specific inhibitors of multidrug resistance in cancer. Semin. Cancer Biol., *8:* 171–182, 1997.

11. Fornage, B. D., Toubas, O., and Morel, M. Clinical, mammographic, and sonographic determination of preoperative breast cancer size. Cancer (Phila.), *60:* 765–771, 1987.

12. Forouhi, P., Walsh, J. S., Anderson, T. J., and Chetty, U. Ultrasonography as a method of measuring breast tumour size and monitoring response to primary systemic treatment. Br. J. Surg., *81:* 223–225, 1994.

13. Helvie, M. A., Joynt, L. K., Cody, R. L., Pierce, L. J., Adler, D. D., and Merajver, S. D. Locally advanced breast carcinoma: accuracy of mammography *versus* clinical examination in the prediction of residual disease after chemotherapy. Radiology, *198:* 327–332, 1996.

14. Herrada, J., Iyer, R. B., Atkinson, E. N., Sneige, N., Buzdar, A. U., and Hortobagyi, G. N. Relative value of physical examination, mammography, and breast sonography in evaluating the size of the primary tumor and regional lymph node metastasis in women receiving neoadjuvant chemotherapy for locally advanced breast carcinoma. Clin. Cancer Res., *3:* 1565–1569, 1997.

15. Rowinsky, E. K., Donehower, R. C., Jones, R. J., and Tucker, R. W. Microtubule changes and cytotoxicity in leukemic cell lines treated with Taxol. Cancer Res., *48:* 4093–4100, 1988.

16. Bhalla, K., Ibrado, A. M., Tourkina, E., Tang, C., Mahoney, M. E., and Huang, Y. Taxol induces internucleosomal DNA fragmentation associated with programmed cell death in human myeloid leukemia cells. Leukemia (Baltimore), *7:* 563–568, 1992.

17. Rowinsky, E. K., and Donehower, R. C. Paclitaxel (Taxol). N. Engl. J. Med., *332.* 1004–1014, 1995.

18. Olah, E., Csokay, B., Prajda, N., Kote-Jarai, Z., Yeh, Y., and Weber, G. Molecular mechanisms in the antiproliferative action of Taxol and tiazofurin. Anticancer Res., *16:* 2469–2478, 1996.

19. Saunders, D. E., Lawrence, W. D., Christensen, C., Wappler, N. L., Ruan, H., and Deppe, G. Paclitaxel-induced apoptosis in MCF-7 breast-cancer cells. Int. J. Cancer, *70:* 214–220, 1997.

20. Milas, L., Hunter, N. R., Kurdoglu, B., Mason, K. A., Meyn, R. E., Stephens, L. G., and Peters, L. J. Kinetics of mitotic arrest and apoptosis in murine mammary and ovarian tumors treated with Taxol. Cancer Chemother. Pharmacol., *35:* 297–303, 1995.

21. Milross, C. G., Mason, K. A., Hunter, N. R., Chung, W., Peters, L. J., and Milas, L. Relationship of mitotic arrest and apoptosis to

antitumor effect of paclitaxel. J. Natl. Cancer Inst., *88:* 1308–1314, 1996.

22. Symmans, W. F., Cangiarella, J. F., Symmans, P. J., Cohen, J. M., Yee, H. T., Bennett, G., Amorosi, E. L., and Waisman, J. Apoptotic index from fine needle aspiration cytology as a criterion to predict histologic grade of non-Hodgkin's lymphoma. Acta Cytol., *44:* 194–204, 2000.

23. Sloop, G. D., Roa, J. C., Delgado, A. G., Balart, J. T., Hines, M. O., III, and Hill, J. M. Histologic sectioning produces TUNEL reactivity. A potential cause of false-positive staining. Arch. Pathol. Lab. Med., *123:* 529–532, 1999.

24. Vagunda, V., Kalabis, J., and Vagundová, M. Correlation between apoptotic figure counting and the TUNEL technique. Anal. Quant. Cytol. Histol., *22:* 307–310, 2000.

25. Cameron, D. A., Gregory, W. M., Bowman, A., and Leonard, R. C. F. Mathematical modelling of tumour response in primary breast cancer. Br. J. Cancer, *73:* 1409–1416, 1996.

26. Au, J. L-S., Li, D., Gan, Y., Gao, X., Johnson, A. L., Johnston, J., Millenbaugh, N. J., Jang, S. H., Kuh, H-J., Chen, C-T., and Wientjes, M. G. Pharmacodynamics of immediate and delayed effects of paclitaxel: role of slow apoptosis and intracellular drug retention. Cancer Res., *58:* 2141–2148, 1998.

27. Moos, P. J., and Fitzpatrick, F. A. Taxanes propagate apoptosis via two cell populations with distinctive cytological and molecular traits. Cell Growth Differ., *9:* 687–697, 1998.

28. Brown, J. M., and Wouters, B. G. Apoptosis, p53, and tumor cell sensitivity to anticancer agents. Cancer Res., *59:* 1391–1399, 1999.

29. Trock, B. J., Leonessa, F., and Clarke, R. Multidrug resistance in breast cancer: a met-analysis of MDR1/gp170 expression and its possible functional significance. J. Natl. Cancer Inst., *89:* 917–931, 1997.

30. Chevillard, S., Pouillart, P., Beldjord, C., Asselain, B., Beuzeboc, P., Magdelénat, H., and Vielh, P. Sequential assessment of multidrug resistance phenotype and measurement of S-phase fraction as predictive markers of breast cancer response to neoadjuvant chemotherapy. Cancer (Phila.), *77:* 292–300, 1996.

31. Ciarmiello, A., Del Vecchio, S., Silvestro, P., Potena, M. I., Carriero, M. V., Thomas, R., Botti, G., D'Aiuto, G., and Salvatore, M. Tumor clearance of technetium 99m-sestamibi as a predictor of response to neoadjuvant chemotherapy for locally advanced breast cancer. J. Clin. Oncol., *16:* 1677–1683, 1998.

32. Mankoff, D. A., Dunnwald, L. K., Gralow, J. R., Ellis, G. K., Drucker, M. J., and Livingston, R. B. Monitoring the response of patients with locally advanced breast carcinoma to neoadjuvant chemotherapy using [technetium 99m]-sestamibi scintimammography. Cancer (Phila.), *85:* 2410–2423, 1999.

33. Haldar, S., Chintapalli, J., and Croce, C. M. Taxol induces *bcl-2* phosphorylation and death of prostate cancer cells. Cancer Res., *56:* 1253–1255, 1996.

34. Fang, G., Chang, B., Kim, C., Perkins, C., Thompson, C., and Bhalla, K. "Loop" domain is necessary for Taxol-induced mobility shift and phosphorylation of bcl-2 as well as for inhibiting Taxol-induced cytosolic accumulation of cytochrome *c* and apoptosis. Cancer Res., *58:* 3202–3208, 1998.

35. Poruchynsky, M. S., Wang, E. E., Rudin, C. M., Blagosklonny, M. V., and Fojo, T. Bcl-xL is phosphorylated in malignant cells following microtubule disruption. Cancer Res., *58:* 3331–3338, 1998.

36. Ibrado, A. M., and Bhalla, K. Temporal relationship of CDK-1 activation and mitotic arrest to cytosolic accumulation of cytochrome *c* and caspase-3 activity during Taxol-induced apoptosis of human AML HL-60 cells. Leukemia (Baltimore), *12:* 1930–1936, 1998.

37. Stewart, Z. A., Mays, D., and Pietenpol, J. A. Defective $G_1$-S cell cycle checkpoint function sensitizes cells to microtubule inhibitor-induced apoptosis. Cancer Res., *59:* 3831–3837, 1999.

38. Perkins, C. L., Fang, G., Kim, C. N., and Bhalla, K. N. The role of Apaf-1, caspase-9, and Bid proteins in etoposide- or paclitaxel-induced mitochondrial events during apoptosis. Cancer Res., *60:* 1645–1653, 2000.

39. Griffon-Etienne, G., Boucher, Y., Brekken, C., Suit, H. D., and Jain, R. K. Taxane-induced apoptosis decompresses blood vessels and lowers interstitial fluid pressure in solid tumors: clinical implications. Cancer Res., *59:* 3776–3782, 1999.

40. Dantzig, A. H., Shepard, R. L., Cao, J., Law, K. L., Ehlhardt, W. J., Baughman, T. M., Bumol, T. F., and Starling, J. L. Reversal of P-glycoprotein-mediated multidrug resistance by a potent cyclopropyldibenzosuberane modulator, LY335979. Cancer Res., *56:* 4171–4179, 1996.

41. Huang, Y., Ray, S., Reed, J. C., Ibrado, A. M., Tang, C., Nawabi, A., and Bhalla, K. Estrogen increases intracellular p26Bcl-2 to p21Bax ratios and inhibits Taxol-induced apoptosis of human breast cancer MCF-7 cells. Br. Cancer Res. Treat., *42:* 73–81, 1997.

42. Haq, R., and Zanke, B. Inhibition of apoptotic signalling pathways in cancer cells as a mechanism of chemotherapy resistance. Cancer Metastasis Rev., *17:* 233–239, 1998.

43. Walczak, H., Miller, R. E., Ariail, K., Gliniak, B., Griffith, T. S., Kubin, M., Chin, W., Jones, J., Woodward, A., Le, T., Smith, C., Smolak, P., Goodwin, R. G., Rauch, C. T., Schuh, J. C. L., and Lynch, D. H. Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand *in vivo*. Nat. Med., *5:* 157–163, 1999.

44. Baselga, J., Norton, L., Albanell, J., Kim, Y-M., and Mendelsohn, J. Recombinant humanized anti-HER2 antibody (Herceptin) enhances the antitumor activity of paclitaxel and doxorubicin against HER2/*neu* overexpressing human breast cancer xenografts. Cancer Res., *58:* 2825–2831, 1998.

45. Lucci, A., Han, T-Y., Liu, Y-Y., Giuliano, A. E., and Cabot, M. C. Multidrug resistance modulators and doxorubicin synergize to elevate ceramide levels and elicit apoptosis in drug-resistant cancer cells. Cancer (Phila.), *86:* 300–311, 1999.

46. Leverkus, M., Neumann, M., Mengling, T., Rauch, C. T., Bröcker, E., Krammer, P. H., and Walzak, H. Regulation of tumor necrosis factor-related apoptosis-inducing ligand sensitivity in primary and transformed human keratinocytes. Cancer Res., *60:* 553–559, 2000.

47. Roh, H., Pippin, J., and Drebin, J. A. Down-regulation of HER2/neu expression induces apoptosis in human cancer cells that overexpress HER2/neu. Cancer Res., *60:* 560–565, 2000.

Clinical Cancer Research

# Paclitaxel-induced Apoptosis and Mitotic Arrest Assessed by Serial Fine-Needle Aspiration: Implications for Early Prediction of Breast Cancer Response to Neoadjuvant Treatment

W. Fraser Symmans, Matthew D. Volm, Richard L. Shapiro, et al.

*Clin Cancer Res* 2000;6:4610-4617.