Determination of Angiogenesis Adds Information to Estrogen Receptor Status in Predicting the Efficacy of Adjuvant Tamoxifen in Node-positive Breast Cancer Patients

Giampietro Gasparini, Stephen B. Fox, Paolo Verderio, Emanuela Bonoldi, Pierantonio Bevilacqua, Patrizia Boracchi, Stefania Dante, Ettore Marubini, and Adrian L. Harris

ABSTRACT

There is experimental and clinical evidence that angiogenesis is involved in breast cancer progression and metastasis. To investigate whether the determination of angiogenesis adds prognostic information to the estrogen receptor (ER) status, we studied a series of 178 node-positive breast cancer patients, with a median follow-up time exceeding 5 years, treated with adjuvant tamoxifen (TAM). We assessed angiogenesis by the quantification of the intratumoral microvessel density and the determination of the Chalkley score using light microscopy. Microvessels were immunostained using the anti-CD31 antibody. The other features studied were ER status and the conventional clinicopathological prognostic indicators. Results were pooled from two collaborating Centers using Chalkley counts to convert intratumoral microvessel density to a common quantification system. We found that Chalkley score was not associated with any other feature studied. In univariate analysis, Chalkley score was significantly predictive of both relapse-free survival (RFS) and overall survival (OS; \( P < 0.00001 \) and \( P = 0.00004 \), respectively). Likewise, ER status, the number of metastatic axillary nodes, histological grading, and tumor size were significantly predictive for RFS and OS. Cox multivariate analysis showed that Chalkley score was the strongest significant independent predictor of outcome. For RFS, ER status, the number of metastatic nodes, and histological grading also retained significance. For OS, the number of metastatic nodes, tumor size, and histological grading were independent prognostic factors. The joint assessment of the above variables had a satisfactory prognostic capability, as found using the Harrel statistics (\( c = 0.77 \)).

These results suggest the validity of using Chalkley counts to assess and compare angiogenesis for prognostic purposes between different Centers. We found that angiogenesis adds significant prognostic information to ER status in predicting the outcome of breast cancer patients treated with adjuvant TAM. In fact, irrespective of the ER status, the patients with highly angiogenic tumors had a poor outcome, even if treated with TAM. For these patients, the inhibition of angiogenesis with specific angioinhibitory drugs may be a promising new therapeutic strategy.

INTRODUCTION

It has been shown that the determination of ER\(^3\) (1, 2) and of some ER-related biological indicators such as progesterone receptor (3, 4) and PS2 protein (5), as well as c-erbB-2 (6) and bcl-2 (7) expression and epidermal growth factor receptor levels (8), is of value to predict the response of breast cancer patients to adjuvant hormone therapy with TAM. However, none of the above markers alone can identify responsive versus nonresponsive tumors (9).

There is biological and pharmacological evidence that angiogenesis could be influenced by endocrine-mediated mechanisms. In hormone-sensitive breast cancer cell lines, the production of some angiogenic peptides, such as transforming growth factor \( \alpha \) (10) and VEGF (11, 12), is under the control of estrogens. Kurebayashi et al. (13) and McLeskey et al. (14) have demonstrated that transfection of the human MCF-7 breast cancer cell line with fibroblast growth factor-4 induces the growth of a highly angiogenic and metastatic cell line (MKS) that is hormone independent and resistant to TAM.

Two recent pilot clinical studies (12, 15), performed in small series of breast cancer patients who received adjuvant TAM, demonstrated that the determination of microvessel density as a measure of angiogenic activity, predicts clinical outcome. However, recently there has been controversy on the role of angiogenesis in breast cancer prognosis (16–20), mainly related to the variability between observers and Centers. Therefore, we performed the present study to test if the two collaborating Centers using different techniques could pool their results and enhance the power of the statistical analysis. An international series of NPBC patients treated with the same schedule of...

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\( ^2 \) To whom requests for reprints should be addressed, at Department of Oncology, St. Bortolo Hospital, 36100 Vicenza, Italy.
adjuvant TAM was assessed to confirm the prognostic value of angiogenesis and the prognostic significance of the 25-point Chalkley eyepiece graticule method.

PATIENTS AND METHODS

Patients

We studied 178 patients with NPBC who had undergone breast cancer surgery at the St. Bortolo Medical Center of Vicenza (n = 113) or at the University of Oxford (n = 65) from June 1987 to June 1992. Criteria of eligibility were: histological T1T1a diagnosis of invasive breast cancer; stages I-III; of histologically confirmed metastases in axillary lymph nodes (stages N1-3, with at least levels I and II cleared, no distant metastasis (M0), unilateral tumor, and no other previous or concomitant invasive tumor. The criteria of selection of the patients for adjuvant hormone therapy were: presence of ER-positive tumors for both pre-, peri-, and postmenopausal patients, or presence of clinical contraindications to administering chemotherapy in patients with ER-negative tumors aged <65 years old (29 cases). TAM was also administered to all the patients aged >65 years old, irrespective of the ER status (24 cases were ER negative). The patient populations of the present study, overall, have different characteristics than the series enrolled in the two pilot studies from the two Centers (12, 15), although there is a partial overlap of cases. In particular, the present series from Oxford includes only the node-positive cases of the previous study (12), but with an updated follow-up. The present series from Vicenza includes 74 cases already studied in the pilot study (15), but 39 new cases were added, microvessel density was converted to Chalkley counts (see below), and, finally, follow-up of the series was updated.

Local surgical treatments were modified radical mastectomy in 73 patients or quadrantectomy with axillary dissection as advocated by Veronesi et al. (21) in 45 patients or lumpectomy in the other 60 patients. In all of the patients treated with conservative surgery, a 5–6-week course of radiation therapy was given within 6 weeks from surgery. Conservative surgery and radiation therapy were performed as an alternative to modified radical mastectomy in those patients with primary tumors less than 3 cm in diameter.

Adjuvant Treatment

All of the patients received, within 4 weeks from surgery, 20 mg TAM in two doses daily for at least 3 years or until tumor progression.

Follow-Up

Physical examination was performed every 4 months in all women. Radiographic studies including chest roentgenogram, mammography, and liver echotomography were carried out every 12 months, or earlier whenever clinically indicated. Hematological tests, including 12-channel biochemical profile and complete blood cell counts, were repeated every 6 months. RFS and the OS were calculated as the period from surgery until the date of the first recurrence (RFS) or death (OS).

Primary treatment failure was defined as the first documented evidence of new disease manifestation(s) in locoregional area(s), distant site(s), or a combination of the above. Any new disease involvement was accurately assessed by clinical, radiological, and, whenever feasible, cytological or histological examination of the site(s) of first relapse.

Histopathological Studies

Surgical specimens were routinely formalin fixed and paraffin embedded. Tumors were classified according to the National Surgical Adjuvant Breast Project (22). Histological grading was classified using the modified Bloom and Richardson’s method, according to Elston and Ellis (23).

All identifiable lymph nodes were histologically examined. The median number of cleared axillary lymph nodes was 16 (range, 9–30) and the median number of those metastatic was 2 (range, 1–30).

Immunohistochemical Studies

Determination of the IMD. For each case, the most representative paraffin block was selected, and 4-μm sections were cut. One H&E-stained section was used to select areas representative of the invasive tumor component. Endothelial cells were immunolabeled with the anti-CD31 monoclonal antibody (clone JC/70; Dako Corp., A/S Glostrup, Denmark) at 1:200 dilution for 12 h at room temperature (24, 25).

Briefly, IMD was determined by light microscopy in the area of most intense vascularization (hot spot) of each tumor. This area was found by scanning the tumor sections at low power (×4 and ×10) by identifying those areas with the highest number of distinct microvessels stained. All slides were evaluated in each Center by two pathologists using a double-headed light microscope simultaneously. Both the investigators had to agree on what constituted a single microvessel before any vessel was included in the count. In case of discordant evaluations, the slides were reviewed by a third experienced pathologist. After counting these fields, a 25-point eyepiece graticule was used over the same tumor region and orientated so that the maximum number of points at ×250 (0.135 mm²) were on or within areas of highlighted vessels. All data was converted to Chalkley counts using the standard set of slides for which the correlation of Chalkley and IMD had been assessed (26). Thus, 103 cases were converted to Chalkley counts based on the correlation, and 65 cases had counts carried out directly by the Chalkley method. The mean of the three microvessel and Chalkley counts was calculated using the modified Bloom and Richardson’s method, according to Elston and Ellis (23).

Estrogen Receptor. ER was assessed in paraffin sections using the H-222 rat monoclonal antibody (Abbott Lab, North Chicago, IL) and immunocytochemistry in the Vicenza series. Positive and negative controls were run in parallel with the sections under investigation. The receptors were classified as follows: negative (−) if less than 10% of the cells showed nuclear reactivity and positive (+) if ≥10% nuclei were immunostained (further subcategorization into three levels of intensity of staining did not improve assessment). ER levels were determined by ELISA (Abbott Lab) in the Oxford series. Tumors were classified ER positive if the values of the ER cytosolic...
protein were ≥10 fmol/mg. It has been shown previously that the immunocytochemistry cutpoints were found to be equivalent to 10 fmol/mg in ligand-binding assay (data not shown).

**Statistical Analysis**

The distribution of Chalkley counts between the two Centers and within the modalities of each of the other variables was compared by the Kolmogorov-Smirnov test. The patterns of OS and RFS were estimated by means of the product limit method (Kaplan-Meier) on the basis of a 5-year follow-up period. The role of each of the prognostic variables (univariate analysis) and their joint effect (multivariate analysis) on OS and RFS was investigated using a Cox regression model.

The Chalkley score was analyzed as a continuous variable to avoid possible drawbacks of using “optimal” cutpoints (27). The use of a continuous variable (X) in a Cox regression model imposes a log-linear relationship between HR and X. To characterize the relationship of the covariate with clinical outcome, we used a regression model based on restricted cubic splines with four nodes located at the quintiles of the distribution of the continuous covariate (28). The significance of the nonlinear relationship was assessed by the likelihood ratio test.

In the Cox regression model, each of the regression coefficients is the logarithm of HR, which is constant in time. Under the null hypothesis that a variable has no prognostic role on RFS or OS, HR is expected to be 1.00. The hypothesis of HR = 1.00 was tested using a Wald statistic. In the multivariate analysis, we adopted an initial model containing the variables that were statistically significant (α = 10%) in the univariate analysis and the interaction terms between Chalkley score and ER because they were retained as clinically relevant. To verify whether the variables had a different prognostic significance between the two Centers, the “Center” was forced as a variable in the multivariate model. A final more parsimonious model was obtained using a backward selection procedure. However, all of the variables that were not statistically significant in univariate analysis were singly added in the regression model so as to avoid not losing an important prognostic factor. Then the variables not statistically significant at the conventional significance of 5% were excluded from the final model. In both univariate and multivariate analyses, the putative better prognosis category was considered as a reference category.

The statistical significance is only a prerequisite for clinical significance; therefore, we tried to measure how much prognostic information the clinico-biological variables actually provide. Therefore, we adopted in the final multivariate Cox regression model, the Harrel c statistic (29) to measure the predictive capability of the model, using the method already reported (30). The null hypothesis (that the variables are not useful discriminators of outcome) is that the value of the above test is equal to 0.5. To be a good discriminator of clinical outcome, the c statistics have to be close to unity. To guide the clinical reader to interpret the value of this statistic, we indicate that a value between 0.70 and 0.80 could be considered as satisfactory, while values >0.80 could be considered to have good predictive capability.

The contribution of each variable to the predictive capability of the model was investigated by comparing the values of the c statistics in the final regression model with those of the same model without the variable itself. Statistical analysis was performed using the Statistical Analysis System package (SAS Institute, Cary, NC).

**RESULTS**

**Clinical Outcome.** Because this is a series of patients treated with adjuvant TAM, biological features are skewed toward features that may be associated with hormone response. As reported in Table 1, the majority of the patients are postmenopausal, with ER-positive and grade I-II tumors. At a median follow-up time of 63 months (range, 0–87), the probability of RFS and OS in the total series was 63.3 and 71.6%, respectively. During the period of observation, disease recurred in 61 patients (dominant metastasis sites in: viscera, n = 21; bone, n = 20; and soft tissues, n = 20), and 49 died. In the survival analysis, we included all causes of death (eight patients died of causes other than breast cancer progression).

**Analysis of Center-to-Center Variability of the Determinations of Microvessel Density.** Some clinicopathological characteristics of the patients were different between the two Centers, but the differences were only of marginal statistical significance. The Oxford patients more frequently were premenopausal (17% versus 8%; χ² = 3.32; P = 0.070) and had a higher frequency of large primaries (pT2,3 tumors: 58% versus 43%; χ² = 3.76; P = 0.055) but had a lower number of involved nodes (± three metastatic axillary nodes: 35% versus 51%).
Predictors of Outcome of Node-positive Breast Cancer

This implies that the prognostic significance of the variables studied, also the variable “Center” and the interaction of this variable with the Chalkley score.

In this model, both the variable “Center” and the interaction between the Center and Chalkley score were not statistically significant (RFS: “Center,” HR = 0.53; χ² = 0.29 and P = 0.58; interaction Center, Chalkley score: HR = 1.24; χ² = 1.54 and P = 0.21; OS: Center, HR = 0.37; χ² = 0.71 and P = 0.39; interaction Center, Chalkley score: HR = 1.14; χ² = 0.54 and P = 0.46). These results indicate that the prognostic significance of Chalkley counts was similar in the two cohorts of cases. Furthermore, because also the variable “Center” is not statistically significant, the prognosis of the patients enrolled in the two Centers was similar when corrected for the clinicobiological characteristics.

**Association of Chalkley Counts with the Other Variables.** The degree of angiogenesis in the overall series of NPBC was not associated with any other variable considered as indicated by the Kolmogorov Smirnov test (KS): menopausal status (post-versus pre-menopausal, KS = 0.55; P = 0.91); histological type (lobular and others versus ductal, KS = 0.35; P = 0.99); tumor size (pT2-3 versus pT1, KS = 0.35; P = 0.99); histological grading (GIII versus GII-I, KS = 0.80; P = 0.53); involved nodes (≥3 versus <3, KS = 0.46; P = 0.98); and ER status (negative versus positive, KS = 0.49; P = 0.96).

**Prognostic Value of Angiogenesis.** We found that Chalkley score was a significant prognostic indicator for RFS and OS (HR = 1.39, P < 0.00001 and HR = 1.32, P = 0.00004, respectively; Fig. 1). As reported in Table 2, the probability of 5-year RFS and OS decreased as the quintiles of the distribution of Chalkley score increased. Thus, 5-year RFS varied from 32.2% in patients with Chalkley counts greater than 6.6 to 83.3% in patients with Chalkley counts less than 2.6.

We analyzed the probability of RFS and OS separately in ER-positive and ER-negative tumors. For RFS, prognosis was strictly influenced by the degree of angiogenesis in both subgroups of patients. However, as expected, patients with ER-positive tumors had a better outcome than those with ER-negative tumors (Fig. 2).

A similar behavior was also observed for OS. Five-year OS was 80.7% in patients with ER-positive tumors compared to 54.9% in those with ER-negative tumors. At parity of Chalkley count value, prognosis of ER-positive patients was always better than that of those with ER-negative cancers (Fig. 3).

**Table 2 Five-year RFS and OS according to the Chalkley score (quintiles)**

<table>
<thead>
<tr>
<th>Chalkley score</th>
<th>No. of cases</th>
<th>No. of relapses</th>
<th>% 5-year RFS</th>
<th>No. of deaths</th>
<th>% 5-year OS</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤2.6</td>
<td>39</td>
<td>6</td>
<td>83.3</td>
<td>5</td>
<td>89.2</td>
</tr>
<tr>
<td>2.7-4.1</td>
<td>33</td>
<td>6</td>
<td>81.5</td>
<td>5</td>
<td>87.9</td>
</tr>
<tr>
<td>4.2-5.0</td>
<td>38</td>
<td>14</td>
<td>61.3</td>
<td>9</td>
<td>74.6</td>
</tr>
<tr>
<td>5.1-6.6</td>
<td>33</td>
<td>12</td>
<td>62.5</td>
<td>11</td>
<td>65.0</td>
</tr>
<tr>
<td>&gt;6.6</td>
<td>35</td>
<td>23</td>
<td>32.2</td>
<td>19</td>
<td>50.0</td>
</tr>
</tbody>
</table>

Total 178 61 49

χ² = 4.23; P = 0.040. The patients of the two Centers also had a non-statistically significant diverse distribution of Chalkley counts; the median and mean values of Chalkley score were 6.3 (range, 2.6 to 9.3) and 5.94 versus 3.6 (range, 1.3 to 9.8) and 3.9, respectively (Kolmogorov Smirnov test, KS = 1.24; P = 0.088). This implies that the prognostic significance of the variables should not be significantly different when evaluated separately in the two cohorts of cases. To verify this latter question, we included into the Cox multivariate analysis model, besides all

![Figure 1](https://example.com/figure1.png)

*A, 5-year RFS (64.4%); patients at risk, n = 68; patients whose cancer recurred, n = 60 and B, 5-year OS (73.8%); patients at risk, n = 88; patients who died, n = 45 according to the Chalkley score (continuous variable) in the overall series.*

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patients whose cancer recurred, other statistically significant predictive variables were: the number of metastatic nodes (HR = 2.15; P = 0.0062); ER status (HR = 1.84; P = 0.028); and histological grading (HR = 1.75; P = 0.047). For OS, the other statistically significant independent predictive variables were: the number of metastatic nodes (HR = 4.44; P = 0.00002); tumor size (HR = 2.12; P = 0.016); and histological grading (HR = 2.02; P = 0.025)(Table 4).

**Predictive Capability of the Clinicopathological Variables.** The overall capability of the variables in the final regression model to predict clinical outcome of the patients was satisfactory (c = 0.77 both for RFS and OS). The contribution of each variable is shown in Table 5. The highest predictive capability was given by Chalkley score. For RFS, the contribution of grading, number of involved nodes, and ER were irrelevant (c = 0.75 compared to c = 0.77), whereas the contribution of Chalkley score was relevant (c = 0.68 versus c = 0.77). Regarding OS, the c value of the model without Chalkley score was 0.70, whereas the contribution of the other three variables was minor.

**DISCUSSION**

In general, the angiogenic activity of a tumor is the consequence of the net balance between angiogenic stimuli and inhibitory pathways (31). To convert to the angiogenic phenotype, a tumor must increase the production of one or more angiogenic peptides (32) and/or decrease the production of one or more natural angiogenesis inhibitors (33, 34).

Angiogenesis plays an important role in breast cancer progression (31, 35, 36). It is known that infiltrating ductal breast carcinoma expresses high levels of VEGF and that its receptors are highly present in endothelial cells of microvessels adjacent to malignant cells (37). Moreover, in human breast cancer, the coexpression of VEGF and platelet-derived growth factor is present in about one-half of the tumors, and they are significantly associated with high vascularization (38). The growth of most breast cancers is under endocrine control mechanisms. Recent studies suggest that angiogenic peptides, which may be released by tumor cells, endothelial cells, macrophages, or from extracellular matrix (31), may be hormonally regulated (39) and that they may influence the hormone response by paracrine mechanisms (10–12, 40, 41).

Because of the above data, we assessed angiogenesis in a series of NPBC patients treated with adjuvant TAM. We found that Chalkley counts presented a nonsignificant different distri-
Table 3 Univariate statistical analysis on 5-Year RFS and OS

<table>
<thead>
<tr>
<th>Variable Category</th>
<th>RFS</th>
<th></th>
<th>OS</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR</td>
<td>CI</td>
<td>(\chi^2)</td>
<td>(P)</td>
</tr>
<tr>
<td>Menopausal status</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Post vs. pre-peri menopausal</td>
<td>0.87</td>
<td>0.41–1.84</td>
<td>0.121</td>
<td>0.72</td>
</tr>
<tr>
<td>Histological type</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lobular and others vs. ductal</td>
<td>0.91</td>
<td>0.47–1.76</td>
<td>0.064</td>
<td>0.79</td>
</tr>
<tr>
<td>Tumor size</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(T_2-3) vs. (T_1)</td>
<td>1.59</td>
<td>0.96–2.65</td>
<td>3.26</td>
<td>0.070</td>
</tr>
<tr>
<td>Grading</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GIII vs. GII–I</td>
<td>2.12</td>
<td>1.23–3.65</td>
<td>7.32</td>
<td>0.0068</td>
</tr>
<tr>
<td>Number of involved nodes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\geq 3) vs. (&lt;3)</td>
<td>1.66</td>
<td>1.00–2.75</td>
<td>3.92</td>
<td>0.047</td>
</tr>
<tr>
<td>ER status</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative vs. positive</td>
<td>2.48</td>
<td>1.49–4.11</td>
<td>12.44</td>
<td>0.0042</td>
</tr>
<tr>
<td>Chalkley score</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Continuous</td>
<td>1.39</td>
<td>1.22–1.57</td>
<td>26.14</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

* Wald statistic.

Table 4 Independent variables in the Cox-multivariate statistical analysis on 5-year RFS and OS (final model)

<table>
<thead>
<tr>
<th>Variable Category</th>
<th>RFS</th>
<th></th>
<th>OS</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR</td>
<td>CI</td>
<td>(\chi^2)</td>
<td>(P)</td>
</tr>
<tr>
<td>Chalkley score</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Continuous</td>
<td>1.42</td>
<td>1.24–1.62</td>
<td>26.98</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Number of involved nodes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\geq 3) vs. (&lt;3)</td>
<td>2.15</td>
<td>1.24–3.73</td>
<td>7.48</td>
<td>0.0062</td>
</tr>
<tr>
<td>Tumor size</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(T_2-3) vs. (T_1)</td>
<td>2.12</td>
<td>1.14–3.93</td>
<td>5.75</td>
<td>0.016</td>
</tr>
<tr>
<td>Grading</td>
<td></td>
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<tr>
<td>GIII vs. GII–I</td>
<td>1.75</td>
<td>1.00–3.06</td>
<td>3.94</td>
<td>0.047</td>
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<td>ER status</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative vs. positive</td>
<td>1.84</td>
<td>1.06–3.19</td>
<td>4.78</td>
<td>0.028</td>
</tr>
</tbody>
</table>

* CI, 95% confidence interval.

Table 5 Prognostic capability of the variables statistically significant in the final regression model by the Harrel c statistic (c)

<table>
<thead>
<tr>
<th>Model</th>
<th>Value of c</th>
</tr>
</thead>
<tbody>
<tr>
<td>RFS</td>
<td>OS</td>
</tr>
<tr>
<td>Without each single variable</td>
<td></td>
</tr>
<tr>
<td>Grading</td>
<td>0.75</td>
</tr>
<tr>
<td>Number of nodes</td>
<td>0.75</td>
</tr>
<tr>
<td>Tumor size</td>
<td>0.76</td>
</tr>
<tr>
<td>ER</td>
<td>0.75</td>
</tr>
<tr>
<td>Chalkley score</td>
<td>0.68</td>
</tr>
<tr>
<td>Full model</td>
<td>0.77</td>
</tr>
</tbody>
</table>

The contribution of angiogenesis was the highest for both RFS and OS (Table 5). These results suggest the validity of the method of Chalkley counts to convert microvessel density into a more objective quantitative assessment. Since the prognostic value of Chalkley score was of borderline statistical significance in pilot studies with small numbers of cases analyzed \(P = 0.07\); Ref. 12), our results also suggest that the clinical significance of the determination of Chalkley score is strengthened by increasing the study size.

We found that the patients treated with adjuvant TAM who have the best prognosis are those with low vascularized, ER-positive, few nodes involved (<3), small primary, and well-to-moderately differentiated tumors. This series is representative of breast cancer cases because the prognostic indicators commonly found to be important in other series with a broader spectrum of breast cancer (1-3) remained significant, including the numbers of involved nodes, tumor size, histological grading, and ER status (the latter for RFS only).

Thus, the results of the present study reinforce preliminary data observed in two pilot studies demonstrating that the determin-
mination of angiogenesis is predictive of the outcome of patients treated with adjuvant TAM (12, 15). In the pilot study from Vicenza (15), it was found that in a series of 84 NPBCs, the determination of vascularization, assessed by the determination of the absolute microvessel density at the “hot spot” of each tumor, significantly predicted the outcome of the patients. The results of the multivariate analysis indicated that also ER status and the number of involved nodes were significant indicators for RFS and OS, respectively. However, the small number of cases did not permit definitive conclusions because some prognostic factors had a borderline statistical value. An independent study by Macaulay et al. (12) assessed angiogenesis by using the Chalkley score in a series of 88 breast cancer patients, with or without axillary nodes involved, followed for a relatively short time. They found that Chalkley score had a borderline prognostic value for OS (P = 0.07) and that the degree of angiogenesis was unrelated to the conventional clinicopathological features. However, it was not possible to establish the prognostic significance of angiogenesis within patients with different characteristics, mainly nodal involvement and ER status.

Although it was suggested that TAM may have an antiangiogenic effect as part of its mechanisms of action (42, 43), this has not been shown in vivo in clinical studies. In an experimental model, Noguchi et al. (43) showed that TAM treatment of ER-positive breast carcinoma down-regulated transforming growth factor α, an angiogenic factor, but did not show that the treatment resulted in a lower microvessel density. Our observation that the tumors with the highest angiogenesis have the worse outcome, even when ER-positive, would suggest that this is not the case.

However, the mechanisms by which high angiogenesis in ER-positive cases is associated with poor response to endocrine therapy is unknown. Several possibilities may be considered. Growth factors produced from the stroma and vessels may stimulate tumor cells directly, such that a paracrine pathway is stimulating tumor growth, and this is able to overcome the effect of TAM. Potential factors released from the stroma could be basic fibroblast growth factor, VEGF, and insulin-like growth factor (44, 45), and the paracrine effect of these factors may be more important to stimulate tumor growth than the mitogenic effect of estrogens. Another possibility is that stromal cells, such as macrophages, produce growth factors that stimulate both the tumor and the endothelium (46), and again this would result in high angiogenesis with hormone resistance. Finally, although the tumors are ER positive, they may have epigenetic or genetic changes that result in TAM resistance and in enhanced angiogenesis. A potential candidate here would be mutations of p53 which may up-regulate angiogenesis and potentially give a hormone-independent growth pathway (36). We also found that the worst outcome among the ER-negative patients was that of the cases with high microvessel density. One could speculate that if angiogenesis is an important ER-unrelated mechanism of action of TAM, the most responsive tumors, in the absence of ER expression, would be those most dependent on angiogenesis, i.e., with high microvessel density. Since we found the opposite, again, our results would suggest that TAM does not have a relevant antiangiogenic effect in vivo.

In conclusion, our study shows the prognostic importance of Chalkley counts as a method of assessing the outcome of patients treated with adjuvant TAM and the robustness of using this technique for multi-Center comparisons and future prospective collaborative studies. Furthermore, our data suggest that the determination of angiogenic activity of breast cancer adds independent prognostic information to ER and that it may be a useful target to identify the patients who gain a benefit from conventional anticancer treatments (with low vascularized tumors) from those poorly responsive (with high vascularized tumors) who may be selected for novel therapeutic strategies, including modulation of angiogenesis.

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Determining of angiogenesis adds information to estrogen receptor status in predicting the efficacy of adjuvant tamoxifen in node-positive breast cancer patients.

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