The Prognostic Value of Angiogenesis by Chalkley Counting in a Confirmatory Study Design on 836 Breast Cancer Patients

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ABSTRACT

This study addresses the prognostic value of estimating angiogenesis by Chalkley counting in breast cancer. A population-based group consisting of 836 patients with operated primary, unilateral invasive breast carcinomas was included from a predefined region and period of time. The median follow-up time was 11 years and 4 months. The microvessels were immunohistochemically stained by antibodies against CD34. The Chalkley count was obtained by a 25-point grid within three, subjectively selected, vascular tumor areas of highest microvessel density. The Chalkley count was analyzed in three categories using predefined Chalkley cutoff points at five and seven. There were significant correlations between high Chalkley counts and axillary lymph node metastasis, large tumor size, high histological malignancy grade, and histological type. A high Chalkley count showed lower probabilities of recurrence-free survival (P < 0.001) and overall survival (P < 0.0001). In the Cox multivariate analysis, the hazard ratio (and 95% confidence interval) showed that the increased risk to die were: 1.55 (1.19–2.03) with Chalkley counts between 5 and 7; 2.26 (1.72–2.98) with counts ≥7 compared with counts ≤5; and 1.46 (1.14–1.87) with counts ≥7 compared with counts between 5–7. The study confirmed that estimation of angiogenesis by Chalkley counting had independent prognostic value in breast cancer patients. The Chalkley count could be useful to stratify node-negative patients for adjuvant treatment.

INTRODUCTION

There is still uncertainty about angiogenesis as a prognosticator in breast cancer, with publications of conflicting results, as reviewed earlier (1). Systematic reviewing of the published results seems, moreover, to be a difficult task, mainly because of heterogeneity in the selection of study population and of the applied techniques. Hence, there is need for a prognostic study, designed to be a large confirmative study of prestated hypotheses, a so-called prognostic Phase III study as suggested by Simon and Altman (2).

The tumor growth dependency on angiogenesis (3) makes the hypothesis of angiogenesis as a prognosticator attractive. The clonal origin of angiogenic activity, being heterogeneously distributed within the tumor, has been used as an argument for quantifying angiogenesis in the areas of the most intense neo-vascularization, the neovascular hot spots (4). Studies of the assessment of angiogenesis have mainly been based on this hot-spot approach, preferentially using the technique of counting microvessel profiles by all immunohistochemically stained distinct endothelial cells or cell clusters in a microscopic field. However, different studies have used different techniques. One of these methods is represented by applying a Chalkley grid with 25 points on the hot spots (5–7). This technique, suggested as a standard in an international consensus (8), is considered to be a simple and acceptable procedure for daily clinical use (9).

Prognosis-related Chalkley studies of breast cancer by Fox et al. (5) described a sample of 109 node-negative patients, who were expanded to 211 patients, including node-positive cases, with a median follow-up of 3 years and 6 months, and 27 deaths (6, 7). We have evaluated the reproducibility of the Chalkley count assay (9). It would be relevant to reevaluate the prognostic value of angiogenesis by Chalkley counting in a confirmative study design (2). In the present study, it was decided to use prestated cutoff points, to increase the number of events and follow-up period, and probably mostly important, to select an inception cohort including all operable primary unilateral invasive breast carcinoma patients in a predefined geographical area and period of time.

The study’s aim was thus to investigate the prognostic value of estimating angiogenesis by Chalkley counting in breast cancer patients, using a large population-based confirmatory study design.

MATERIALS AND METHODS

Patients. The investigation was based on the complete population of patients with the diagnosis of breast cancer from a certain geographical area. The region was the primary catchment area of Odense University Hospital, where the patients underwent surgery from January 1, 1980 to December 31, 1990. Mammographic screening for breast cancer was not performed in the background population during this period. The inclusion criterion was patients with a primary, unilateral, and operable...
invasive breast carcinoma. The exclusion criteria were patients with distant metastasis at the time of diagnosis, locally advanced disease, inflammatory carcinoma, synchronous bilateral breast cancer, and a diagnosis of carcinoma in situ. Women with previous malignant disease, apart from carcinoma in situ of the uterine cervix or skin cancer, were also excluded, and women who did not have axillary dissection with at least one lymph node removed. The median number of lymph nodes removed at the axillary dissection was 10 (range, 1–48). Surgery, adjuvant chemotherapy, and radiotherapy were carried out according to the nationwide recommendations of the Danish Breast Cancer Cooperative Group (10). The inclusion criterion selected 841 of the 1252 women who were admitted with the diagnosis breast cancer. No tumor tissue was left for immunohistochemical staining in five patients, leaving 836 patients for analysis.

Follow-Up. For all patients, clinical and pathological records were reviewed. Patients were followed regularly for 10 years at the Odense University Hospital, according to the Danish Breast Cancer Group recommendation (10). Some older patients were followed by their general practitioner and only referred to the hospital if recurrence was suspected. Twenty patients moved to other parts of the country. For those patients, the departments providing the follow-up were contacted, and follow-up information was obtained from clinical records. Two patients moved out of the country and were lost to follow-up; they were censored at the time of last contact. All of the other patients were followed until death or the study closing date of October 31, 1996. The recruitment of patients took place during 11 years and further observation during the next 5 years and 10 months. The maximum possible observed survival period for the initial and last patients was therefore 16 years and 10 months, and 5 years and 10 months, respectively. The potential median follow-up time was 11 years and 4 months (136 months).

End Points. The prognostic analyses were carried out regarding RFS and OS. The corresponding end points were the first recurrence at any site (RFS) or death from any cause (OS). Of the 836 patients, 312 had recurrence, and 381 died.

Immunohistochemistry. For each tumor, all archival tumor blocks were initially checked by H&E stained sections to select a tumor block with an invasive carcinoma, including the tumor border and as large a cross-sectional area as possible. One 4-μm-thick section from each formalin-fixed and paraffin-embedded tumor was mounted on a ChemMate slide (Dako, Glostrup, Denmark). Epitope retrieval for CD34 was performed by microwave heating in 10 mM Tris plus 0.5 mM EGTA buffer (pH 9). Three containers, each with 50 slides in 225 ml of buffer, were placed on the edge of a turntable inside the microwave oven. The slides were heated for 25 min at a power setting of 600 W, cooled in the buffer for 15 min, and rinsed in water for 5 min. The immunostaining procedure was automated, using the ChemMate Peroxidase/DAB kit on the TechMate 1000 instrument. As primary antibody against CD34, we used clone QBEnd/10 (NovoCastra, Newcastle, United Kingdom) diluted 1:20 with overnight incubation at 4°C. The primary antibody against the estrogen receptor was clone ER1D5 (Dako) diluted 1:200 with overnight incubation at 4°C, which was preceded by epitope retrieval by microwave heating with citrate buffer (pH 6.0). Negative controls were produced by omitting the primary antibody, and for each slideholder, a positive control was produced by adding a section with numerous vessel profiles and a section that was estrogen receptor positive.

The histological type of breast tumor was determined according to the WHO guidelines (11). Histological malignancy grading followed the grading system of Bloom and Richardson (12). Tumor size was measured in millimeters by the pathologist as the largest diameter of the invasive carcinoma. Estrogen receptor status was determined as positive when >10% of the tumor cells were positive.

Estimating Angiogenesis by Chalkley Counting. The Chalkley counting procedure has been described in detail earlier (5, 6). Briefly, the three most vascular areas (hot spots) with the highest number of microvessel profiles were chosen subjectively from each tumor section. A 25-point Chalkley eyepiece graticule (13) was applied to each hot-spot area and oriented to permit the maximum number of points to hit on, or within the areas of immunohistochemically highlighted microvessel profiles (Leitz Orthoplan, ×250; Chalkley grid area, 0.196 mm²). The Chalkley count for an individual tumor was taken as the mean value of the three graticule counts. All Chalkley counts were performed by one observer, which represents a modification of the procedure described by Fox et al. (5, 7), in which two observers used a conference microscope. Angiogenesis was estimated without knowledge of the clinical data or prognostic outcome. Less than 5 min were used for assessment of each tumor. A satisfying reproducibility of the Chalkley assay was reported in an earlier investigation (9). The prognostic analyses were based on categorical Chalkley count cutoff points at 5 and 7, as defined in earlier studies (6, 7). Fig. 1 shows examples of hot spots with a superimposed Chalkley grid with low, intermediate, and high Chalkley counts, respectively.

Statistics. The predetermined cutoff points from the earlier study were used for the prognostic analyses (6). The sample size was chosen to assure a reasonable number of events, which were expected to be ~55% for crude survival after 10 years of follow-up (14). A suggested calculation (15) of the required number of events is given by: \( \frac{(z_{1-\alpha} + z_p)^2}{w(\ln(HR))^2} \times w^2 \), where \( z_{1-\alpha} \) and \( z_p \) are 1.64 and 1.28 with 5% significance level and 90% statistical power, and \( HR \) is the hazard ratio, expected to be, for example, 1.7 for the highest Chalkley category, and \( w \) is the prevalence of the poor risk factor, expected to be one-third, in that the predetermined cutoff points were tertiles. Using this equation, the required number of events should be 169 and the sample size at least 307. Others have suggested that the number of events should be at least 10 times the total number of variables included (2, 16, 17). With respect to RFS, we have 312 events allowing us to fit reasonably models with ~30 parameters, which is smaller than the maximal number of parameters considered in one model in this report.

The associations of the Chalkley count with other clinicopathological parameters was tested by the Spearman correlation test for ordinal variables and the \( \chi^2 \) test for nominal variables. The univariate relationship between prognostic variables and follow-up end points was illustrated by Kaplan-Meier plots for.

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3 The abbreviations used are: RFS, recurrence-free survival; OS, overall survival; CI, confidence interval.
survival probabilities (18). The differences between survival functions were compared by the log-rank test. The multivariate relationship was evaluated by the Cox proportional hazard regression analysis (19). Proportional hazard rates were graphically controlled by log-minus-log survival plots from the multivariately analyzed data stratified by the controlled variable. The estrogen receptor status did not have proportional hazard rates to fulfill the assumption for the Cox model; hence, we stratified the Cox models by estrogen receptor status. The Cox models were developed by the backward selection procedure, using a removal limit based on the 10% \( P \) from the likelihood ratio statistics. The risk of the categorical covariates was estimated in relation to the reference category, which was always the “lowest” category. Age was an exception, in that the reference was the 40–49 age group, because the category of patients <40 years was rather limited in number. To investigate the shape of the relationship between the risk of relapse and the Chalkley count, we recorded the variable into 10 groups by \( \frac{10}{10} \) percentiles. The Chalkley variable with 10 categories was then reintroduced to the previously developed final Cox model. To investigate a possible time-dependent effect of the variable Chalkley count, an additional Cox model was considered, including the interaction between time and this variable. Calculations were performed with SPSS 7.5 (SPSS, Inc.)

RESULTS

Clinical Data. Table 1 shows the distribution of clinico-pathological data of the patients in the study population and the association between the Chalkley count categories and the different characteristics. Actual tertiles of the Chalkley estimates were 5.0 and 6.67, but the preselected cutoff points at 5 and 7 were used. Of the 836 patients, 337 (40%) had Chalkley count \( \leq 5 \), 259 (31%) between 5 and 7, and 240 (29%) \( \geq 7 \). The median Chalkley count was 5.67 (range, 2.33–13.0), and mean 6.0 (SD 1.8). A high Chalkley count was significantly associated with a high number of axillary lymph node metastases, large tumor size, high histological malignancy grade, and estrogen receptor-negative tumors. Moreover, a significant association with histological type was seen in that lobular carcinomas had low Chalkley counts. There was a significant association with type of surgery, where the significantly smaller lumpectomized tumors had lower Chalkley counts. High age and postmenopausal patients had tumors that tended to have higher Chalkley counts, although these associations were not significant. None of the adjuvant treatment modalities were significantly associated to the Chalkley count.

Univariate Analysis. The univariate analysis showed that high Chalkley count was a significant prognostic indicator of poor outcome for both RFS and OS (Fig. 2), \( P < 0.0001 \). This relation was also found in the node-negative (\( P < 0.0001 \)) and node-positive (\( P < 0.0001 \)) patients. The 5- and 10-year survival probabilities for node-negative, node-positive, and all patients are listed in Table 2 for each Chalkley count category.

Apart from the Chalkley count, some other variables also showed prognostic impact. The univariate analysis demonstrated that the prognosis was significantly worsened by a high number of axillary metastatic nodes, \( P < 0.0001 \) for both RFS and OS), large tumor size (\( P < 0.0001 \), both RFS and OS), high

Fig. 1 Examples of Chalkley counts in hot spots with low (a), intermediate (b), and high (c) numbers of vessel profiles. The histological section is moved or the ocular Chalkley grid rotated until the maximal number of dots of the Chalkley graticule hits stained vessel profiles. This is done in three of the apparently most vascular areas (hot spots) for each tumor. The average number of hitting dots from the three hot spots is the Chalkley count of that tumor. Hitting Chalkley dots are marked by red circles. The Chalkley grid area is 0.196 mm\(^2\); \( \times 250 \).
Table 1 Description of clinicopathological data and associated distribution of Chalkley counts

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>All Chalkley counts</th>
<th>Association</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>≤5 (40)</td>
</tr>
<tr>
<td>All patients</td>
<td>836 (100)</td>
<td>337 (40)</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;40</td>
<td>52 (6)</td>
<td>25 (48)</td>
</tr>
<tr>
<td>40–49</td>
<td>182 (22)</td>
<td>71 (39)</td>
</tr>
<tr>
<td>50–59</td>
<td>197 (23)</td>
<td>82 (42)</td>
</tr>
<tr>
<td>60–69</td>
<td>199 (24)</td>
<td>80 (40)</td>
</tr>
<tr>
<td>≥70</td>
<td>206 (25)</td>
<td>79 (38)</td>
</tr>
<tr>
<td>Menopausal status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Premenopausal</td>
<td>315 (38)</td>
<td>137 (44)</td>
</tr>
<tr>
<td>Postmenopausal</td>
<td>521 (62)</td>
<td>200 (38)</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>400 (48)</td>
<td>180 (45)</td>
</tr>
<tr>
<td>1–3</td>
<td>277 (33)</td>
<td>116 (42)</td>
</tr>
<tr>
<td>≥4</td>
<td>159 (19)</td>
<td>41 (26)</td>
</tr>
<tr>
<td>Tumor size (mm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤20</td>
<td>420 (50)</td>
<td>204 (48)</td>
</tr>
<tr>
<td>21–50</td>
<td>361 (43)</td>
<td>116 (32)</td>
</tr>
<tr>
<td>&gt;50</td>
<td>55 (7)</td>
<td>17 (30)</td>
</tr>
<tr>
<td>Histological malignancy grade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>140 (17)</td>
<td>81 (58)</td>
</tr>
<tr>
<td>II</td>
<td>316 (38)</td>
<td>139 (44)</td>
</tr>
<tr>
<td>III</td>
<td>251 (30)</td>
<td>48 (19)</td>
</tr>
<tr>
<td>Other</td>
<td>129 (15)</td>
<td>69 (53)</td>
</tr>
<tr>
<td>Histological type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ductal</td>
<td>709 (85)</td>
<td>269 (38)</td>
</tr>
<tr>
<td>Lobular</td>
<td>91 (11)</td>
<td>52 (57)</td>
</tr>
<tr>
<td>Special</td>
<td>36 (4)</td>
<td>16 (44)</td>
</tr>
<tr>
<td>Estrogen receptor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>198 (24)</td>
<td>33 (17)</td>
</tr>
<tr>
<td>Positive</td>
<td>638 (76)</td>
<td>304 (47)</td>
</tr>
<tr>
<td>Surgery</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mastectomy</td>
<td>724 (87)</td>
<td>280 (39)</td>
</tr>
<tr>
<td>Lumpectomy</td>
<td>112 (13)</td>
<td>57 (51)</td>
</tr>
<tr>
<td>Adjuvant treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>162 (19)</td>
<td>60 (37)</td>
</tr>
<tr>
<td>Endocrine therapy</td>
<td>139 (17)</td>
<td>51 (37)</td>
</tr>
<tr>
<td>Chemo + endocrine</td>
<td>32 (4)</td>
<td>9 (28)</td>
</tr>
<tr>
<td>Radiation therapy</td>
<td>350 (42)</td>
<td>146 (42)</td>
</tr>
</tbody>
</table>

a Spearman’s correlation test for the ordinal variables and the χ²-test for the nominal clinicopathological variables associated with the Chalkley count categories. Median (range): age, 59 years (24–93 years); positive lymph nodes, 2 (1–47); and tumor size, 20 mm (1–120 mm).

Multivariate Analysis. The initial Cox model, including all of the variables presented in Table 1, showed that the Chalkley count had a significant overall prognostic value (P < 0.0001), being only second to axillary lymph node metastases in prognostic strength. From the initial model, the hazard ratio (and 95% CI) indicates that the risk of dying was 1.55 (1.19–2.03) higher with Chalkley counts between 5 and 7 and 2.26 (1.72–2.98) higher with Chalkley counts ≥7, compared with Chalkley counts ≤5 and 1.46 (1.14–1.87) higher risk with Chalkley counts ≥7, compared with Chalkley counts between 5 and 7 in the primary breast carcinoma. The corresponding risks of recurrence were 1.79 (1.33–2.41), 2.60 (1.91–3.55), and 1.45 (1.11–1.91), respectively.

Table 3 shows the final model from the Cox multivariate analysis, containing the parameters with significant independent prognostic values. The variables that were excluded from the initial model during the backward selection procedure were: menopausal status (pre versus post), histological type (ductal versus lobular or special), adjuvant systemic treatment (none versus given), and radiation therapy (none versus given). The Chalkley count had significant independent prognostic value both for the RFS and OS. The risk of dying was 1.57 higher with Chalkley counts between 5 and 7 and 2.25 higher with Chalkley counts ≥7, compared with having Chalkley counts ≤5 in the primary breast carcinoma. There was also independent prognostic value with respect to both RFS and OS, with increased risk of poor outcome from high number of axillary metastatic nodes, large tumor size, high histological malignancy grade, and high age. Age <40 years was estimated to worsen the prognosis, compared with the 40–49 age group, although not significantly.

The independent prognostic value of the Chalkley count persisted in the subset of node-negative patients, but the risk
estimates of the middle and highest Chalkley count categories were almost the same. Analyzing RFS, the hazard ratio (and 95% CI) for the middle category was 2.40 (1.47–3.92) and for the highest category, 2.23 (1.29–3.88). Analyzing OS, the hazard ratio (and 95% CI) for the middle category was 1.75 (1.16–2.65), and for the highest category, 1.71 (1.09–2.69). Apart from age, histological malignancy grades II and III were the only other significant prognostic factors to predict

### Table 2

<table>
<thead>
<tr>
<th>Chalkley counts</th>
<th>No. of patients</th>
<th>RFS (%)</th>
<th>OS (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5-year</td>
<td>10-year</td>
<td>5-year</td>
</tr>
<tr>
<td>≤5</td>
<td>337</td>
<td>84 ± 2</td>
<td>73 ± 3</td>
</tr>
<tr>
<td>5–7</td>
<td>259</td>
<td>66 ± 3</td>
<td>55 ± 3</td>
</tr>
<tr>
<td>≥7</td>
<td>240</td>
<td>53 ± 3</td>
<td>48 ± 3</td>
</tr>
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</table>

**Node-negative patients (n = 400)**

<table>
<thead>
<tr>
<th>Chalkley counts</th>
<th>No. of patients</th>
<th>RFS (%)</th>
<th>OS (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5-year</td>
<td>10-year</td>
<td>5-year</td>
</tr>
<tr>
<td>≤5</td>
<td>180</td>
<td>89 ± 2</td>
<td>85 ± 3</td>
</tr>
<tr>
<td>5–7</td>
<td>120</td>
<td>73 ± 4</td>
<td>65 ± 5</td>
</tr>
<tr>
<td>≥7</td>
<td>100</td>
<td>68 ± 5</td>
<td>65 ± 5</td>
</tr>
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</table>

**Node-positive patients (n = 436)**

<table>
<thead>
<tr>
<th>Chalkley counts</th>
<th>No. of patients</th>
<th>RFS (%)</th>
<th>OS (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5-year</td>
<td>10-year</td>
<td>5-year</td>
</tr>
<tr>
<td>≤5</td>
<td>157</td>
<td>78 ± 3</td>
<td>60 ± 4</td>
</tr>
<tr>
<td>5–7</td>
<td>139</td>
<td>60 ± 4</td>
<td>46 ± 5</td>
</tr>
<tr>
<td>≥7</td>
<td>140</td>
<td>41 ± 4</td>
<td>37 ± 4</td>
</tr>
</tbody>
</table>

Fig. 2 Probabilities of RFS (above) and OS (below) for the three Chalkley count categories analyzed for all patients (left) and isolated for subgroups of the node-negative (middle) and the node-positive (right) patients.
OS in the node-negative patients. By making the same calculations in the node-positive subgroup, the hazard ratio (and 95% CI) for RFS for the middle Chalkley count category was 1.54 (1.06–2.22), and for the highest category, 2.62 (1.80–3.81). Analyzing OS, the hazard ratio (and 95% CI) for the middle category was 1.47 (1.04–2.07), and for the highest category, 2.60 (1.83–3.68). The histological malignancy grade had no significant prognostic value, whereas both number of metastatic lymph nodes and tumor size, in addition to age, were of significant independent prognostic value in the node-positive patients.

**Linearity of Risk Estimates.** Fig. 3 illustrates the relation between the Chalkley count and the risk of recurrence. A roughly linear relationship can be observed up to a Chalkley count of 7, but for higher values, the risk remains on a roughly constant level. Almost the same shape of the relationship is illustrated between the Chalkley count and the risk of dying (Fig. 4). The deviation from linearity we can observe in Figs. 3 and 4 could be established also by fitting a model with a quadratic term for the effect of the Chalkley count, where the coefficient of the quadratic term was significant ($P = 0.002$, RFS; $P = 0.003$, OS).

**Time Dependency of Risk Estimates.** A model with a time-dependent effect of the Chalkley count indicates a time-dependent hazard ratio of $\exp(0.710 - 0.0277 \times t)$ for the difference between counts 5–7 and counts $\leq 5$, and of $\exp(0.5242 - 0.0597 \times t)$ for the difference between counts $\geq 7$ and counts 5–7. This indicates a decrease of the prognostic value of angiogenesis over time. However, both time effects are not significant, showing 95% CI of $-0.126$ to 0.070 and from $-0.170$ to 0.051, respectively.

**DISCUSSION**

The Chalkley count is the number of grid points that hit stained vessels, taken as the average from the assessment of three hot spots. Although intending to be a surrogate estimate of the microvessel density, it is actually a relative area estimate of immunostained vessels expressed by an index without unit. However, the area estimate may biologically represent an interesting parameter regarding tumor-to-blood exchange capacity or the paracrine effect of the vessels.

**Table 3** The Cox multivariate analysis estimated the independent prognostic value by hazard ratios (HR) and 95% CI for RFS and OS. The significant variables are presented as indicators with risk estimates relative to the basic category, where the risk is set to one, e.g., for age the basic category is 40–49 years. $P$s for the single variables are included at ends of baseline category (n = 836).

<table>
<thead>
<tr>
<th></th>
<th>RFS</th>
<th></th>
<th>OS</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$P$</td>
<td>HR (95% CI)</td>
<td>$P$</td>
<td>HR (95% CI)</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40–49 yr</td>
<td>0.0222</td>
<td>1.00</td>
<td>&lt;0.0001</td>
<td>1.00</td>
</tr>
<tr>
<td>$&lt; 40$ yr</td>
<td>1.60</td>
<td>(0.96–2.66)</td>
<td>1.04</td>
<td>(0.57–1.90)</td>
</tr>
<tr>
<td>50–59 yr</td>
<td>1.67</td>
<td>(1.18–2.36)</td>
<td>1.92</td>
<td>(1.33–2.76)</td>
</tr>
<tr>
<td>60–69 yr</td>
<td>1.72</td>
<td>(1.22–2.43)</td>
<td>2.28</td>
<td>(1.59–3.27)</td>
</tr>
<tr>
<td>$\geq 70$ yr</td>
<td>1.42</td>
<td>(0.99–2.03)</td>
<td>4.31</td>
<td>(3.08–6.03)</td>
</tr>
<tr>
<td><strong>Lymph node metastasis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>&lt;0.0001</td>
<td>1.00</td>
<td>&lt;0.0001</td>
<td>1.00</td>
</tr>
<tr>
<td>1–3</td>
<td>1.42</td>
<td>(1.08–1.88)</td>
<td>1.39</td>
<td>(1.08–1.78)</td>
</tr>
<tr>
<td>$\geq 4$</td>
<td>3.54</td>
<td>(2.63–4.75)</td>
<td>3.47</td>
<td>(2.66–4.53)</td>
</tr>
<tr>
<td><strong>Tumor size</strong></td>
<td>0.0008</td>
<td>1.12</td>
<td>0.0035</td>
<td>1.10</td>
</tr>
<tr>
<td>per cm</td>
<td></td>
<td>(1.05–1.19)</td>
<td>(1.03–1.17)</td>
<td></td>
</tr>
<tr>
<td><strong>Histological malignancy</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade I</td>
<td>0.1523</td>
<td>1.00</td>
<td>0.0290</td>
<td>1.00</td>
</tr>
<tr>
<td>Grade II</td>
<td>1.56</td>
<td>(1.03–2.37)</td>
<td>1.59</td>
<td>(1.09–2.31)</td>
</tr>
<tr>
<td>Grade III</td>
<td>1.66</td>
<td>(1.05–2.62)</td>
<td>1.88</td>
<td>(1.24–2.85)</td>
</tr>
<tr>
<td>Nonductal</td>
<td>1.63</td>
<td>(1.00–2.64)</td>
<td>1.50</td>
<td>(0.97–2.32)</td>
</tr>
<tr>
<td><strong>Chalkley counts</strong></td>
<td>&lt;0.0001</td>
<td>1.00</td>
<td>0.0001</td>
<td>1.00</td>
</tr>
<tr>
<td>$\leq 5$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5–7</td>
<td>1.79</td>
<td>(1.33–2.39)</td>
<td>1.57</td>
<td>(1.20–2.04)</td>
</tr>
<tr>
<td>$\geq 7$</td>
<td>2.55</td>
<td>(1.88–3.46)</td>
<td>2.25</td>
<td>(1.71–2.95)</td>
</tr>
</tbody>
</table>

**Fig. 3** Relationship between the Chalkley count and the risk of recurrence. $n = 836$. Bars, SE.
contribution to the methodological variation tends to be lower than for the more frequently used microvessel density method. The present study confirms the independent prognostic value of angiogenesis by the Chalkley method, as preliminary studies have reported (5, 6) in the investigation of breast cancer patients. The earlier studies analyzed the survival data with cutoff points at Chalkley counts 5 and 7, splitting at the tertiles. An increased risk of dying was reported, estimated with a hazard ratio at 1.7 (95% CI, 0.9–2.9), by going from a Chalkley count <5, to between 5 and 7, and to >7. The distribution of the Chalkley counts was the same in our study as in earlier studies, with a general median of 5.67, and a range of 2.33–11.33 in the earlier investigations and 2.33–13.00 in our study. As in the earlier studies, we found that a high Chalkley count significantly correlated with the number of axillary lymph node metastases but in addition, also to tumor size and histological malignancy grade. However, our population of patients included a lower number of large-size tumors and tumors of histological malignancy grade II.

The independent prognostic estimate demonstrated a 57% higher risk of dying when a tumor had a Chalkley count between 5 and 7, and 125% higher risk with Chalkley counts ≥7, compared with the risk associated with tumors showing Chalkley counts ≤5. This is comparable with the 70% increased risk from one Chalkley category to the next, as reported earlier (6). As in the earlier studies, we found that the nodal status was of considerable prognostic impact, independent of the Chalkley count, indicating that these factors should not replace each other but should be used simultaneously for optimal prognostic stratification.

Because it was our aim to confirm prestated hypotheses, the categorization published previously of the Chalkley count with three categories was used. To avoid the assumption about linear increase of the risk between categories, the Chalkley count categories were introduced in relation to the lowest count category in the Cox multivariate analysis. This linearity problem may exist, as suggested by Fig. 3 in the study by Fox et al. (6), in which the survival probabilities from the categories with counts ≤5 and counts between 5 and 7 were almost the same. This was not the case when analyzing all of the patients in the present study. However, the relationship between the uncategorized count and the risk of relapse was also evaluated. The results indicated that a pure linear approach was not adequate to describe the relationship and that it would overestimate the prognostic effect of Chalkley counts ≥8. Hence, also from this point of view, categorization in three levels seems to be a good compromise.

A possible time-dependent effect of angiogenesis estimated by Chalkley counts was investigated. The results indicate that the effect of angiogenesis may decrease with time, although not significantly. This corresponds to the findings for other tumor-related prognostic factors (20).

Regarding the node-negative patients, the risk of dying was almost the same whether the tumor had a Chalkley count of 5–7 or ≥7. This could be interpreted as follows. If the tumor has not metastasized to the axilla, the crucial prognostic step is whether the tumor may reach some kind of a biological angiogenic limit, which may promote progression to metastatic disease. The survival probabilities for node-negative patients with tumor Chalkley counts 5–7 or ≥7 were roughly the same as for node-positive patients with Chalkley counts ≤5. Within the node-positive patients, the risk of dying increased progressively with increasing Chalkley count. This indicates that if the tumor has the ability to metastasize, an increasing vascular component would progressively facilitate this process. This concerted action may be reflected by the histological malignancy grade in the node-negative patients, whereas in the node-positive patients, the extent of metastatic nodal involvement and the tumor size may be biological reflections of the accentuated neovascularization.

In this study population, 92% of the node-negative patients have not received any systemic adjuvant treatment. The survival probabilities of the node-negative patients with Chalkley counts 5–7 or ≥7 were not better than for the node-positive patients with Chalkley counts ≤5, who usually receive adjuvant systemic treatment. Hence, the group of node-negative patients with Chalkley counts >5 is of potential interest for evaluating the effect of systemic adjuvant treatment. Furthermore, the vessel estimate may be suitable for stratification for antiangiogenic treatment. At first, it seemed reasonable to give antiangiogenic treatment to highly angiogenic tumors. To the contrary, however, low angiogenic tumors, otherwise having the ability to metastasize, may be more sensitive to antiangiogenic treatment if they are in an early phase before switching to a more aggressive angiogenic phenotype. All treatment efforts should optimally be done in a randomized clinical study, after an additional prognostic stratification based on the Chalkley count, to evaluate whether the patients in the study arm obtain a significant improvement in survival outcome (21).

In conclusion, we have confirmed the importance of the Chalkley count as an independent prognostic factor in breast cancer, together with age, axillary metastatic nodal involvement, tumor size, and histological grade of malignancy. The Chalkley assay can easily achieve a more general application, because it is easy to perform and has satisfying reproducibility, although continuous quality control should be performed under the auspices of established breast cancer organizations. The prognostic
value confirmed in this study should be tested independently under circumstances more closely related to the daily pathological service. The Chalkley count then may be useful to stratify node-negative patients for adjuvant treatment. However, the crucial point will be the future benefit for the patients from the therapeutic implication after the new stratification, based on the additional prognostic value of the Chalkley count.

ACKNOWLEDGMENTS

We thank Stephen B. Fox for giving instruction in the Chalkley technique. The technical assistance of Ole Nielsen and Maj-Britt Berg is greatly appreciated.

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The Prognostic Value of Angiogenesis by Chalkley Counting in a Confirmatory Study Design on 836 Breast Cancer Patients

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*Clin Cancer Res* 2000;6:139-146.

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