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Angiogenesis as Determined by Computerised Image Analysis and the Risk of Early Relapse in Women with Invasive Ductal Breast Carcinoma

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The purpose of this study was to define the value of angiogenesis as a prognostic factor indicating early relapse. We assessed the relationship between parameters of angiogenesis (microvessel count - MVC, microvessel area - MVA and microvessel perimeter - MVP) and relapse-free survival at 50 months in 226 women with invasive ductal breast carcinoma. Anti CD31 antibody was used as a marker of endothelial cells. Microvessel density was measured according to Weidner et al. [23] using a computerised image analysis. The mean parameters of angiogenesis were significantly higher in women with relapse than in those without recurrence within 50 months after surgery. In node negative patients relapse did not occur if MVC was below 38.7. In node-negative subgroup with grade II carcinomas parameters of angiogenesis in the primary tumour differed significantly depending on the presence or absence of relapse. Univariate and multivariate analysis showed the prognostic value of angiogenesis parameters in all study groups with respect to 50-month relapse-free survival. In node negative subgroup only 62.5% of women with tumors with high MVC and as much as 94.9% with low MVC survived 50 months without recurrence. In the Cox analysis of node-negative subgroup only MVC, MVP and MVA were the independent prognostic factors. In women with node-negative disease the evaluation of angiogenesis can identify a subgroup of patients with high risk of relapse, hence it may help in decisions concerning adjuvant therapy. Computerised image analysis is a good and objective technique for evaluating the intensity of angiogenesis in breast cancer.

Introduction

Breast carcinoma is the most frequent malignancy in women. In 1996 morbidity and mortality rates in Poland were 35.9/100,000 and 16.1/100,000, respectively [24]. Therefore it is not surprising that the search for new prognostic factors continues. It is of special importance in women with node-negative breast cancer, as it is known that about one third of them will have recurrences [17]. Good prognostic factors should identify women with good initial prognosis who do not require additional systemic adjuvant therapy [15]. Angiogenesis could be such a factor. Weidner et al. [22, 23] have demonstrated that microvessel density was an indicator of poor prognosis also in women with node-negative breast cancer. Furthermore they found out that microvessel density was the strongest independent prognosticator both for relapse-free survival (RFS) and overall survival (OS) [22]. Conflicting results have been published with regard to prognostic significance of angiogenesis in breast cancer with reports confirming [4, 8, 9, 11, 14, 19], and denying the prognostic value of this factor [16, 21]. Heterogeneity of tumours and study populations as well as lack of standarisation of methods of quantification of angiogenesis and various antibodies used to identify endothelial cells may be responsible for discrepancies of results [20]. In most papers angiogenesis was evaluated in one group, which included ductal breast carcinomas and other histological types which have *a priori* better prognosis than ductal carcinoma. A relatively small number of papers dealt with invasive ductal breast carcinoma only [1 - 3, 7, 12, 13, 18].

The purpose of the present study was to assess the value of angiogenesis as prognostic factor indicating early relapse in women with invasive ductal breast carcinoma. To this end we carried out our study in a homogenous group of ductal invasive breast carcinomas using the anti CD31 antibody [20] and we compared the results with relapse-free survival at 50 months. In order to improve the objectivity and reliability of the measurements, and because it is not known whether microvessel area and perimeter are better prognostic parameters than microvessel count in breast carcinoma, we applied computerised image analysis to assess microvessel count, area and perimeter.

Material and Methods

The study material consisted of tissue samples fixed in 10% buffered formalin and embedded in paraffin, obtained

TABLE 1
Basic clinical and morphological data in women with ductal breast carcinoma

Parameter	n
Age	
≤50	109
>50	117
Tumour diameter*	
≤20	158
>20	68
Histological grade	
I°	18
II°	110
III°	98
Lymph nodes	
with metastases	111
without metastases**	115

*tumour diameter from 4 to 48 mm
**from 1 to 30 lymph nodes with metastases

from primary unilateral invasive ductal carcinoma (NOS) of the breast in 226 women (ranging in age from 26 to 79 years, mean age 53.2 years), in whom mastectomy with axillary lymph nodes removal was performed at the Regional Oncological Hospital in Szczecin between 1994 and 1997. Mastectomy plus axillary lymphadenectomy was performed in 199 women, breast conservation surgery in 27. No therapy was used before the surgery. Adjuvant chemotherapy was used in 63 cases, tamoxifen in 54 women, radiotherapy in 6 cases. In 44 cases chemotherapy was combined with hormonal therapy, in 22 with radiotherapy. In 7 cases hormonotherapy was combined with radiotherapy and in 7 cases all the three modes were used. No adjuvant therapy was applied in 11 women, whereas no data concerning adjuvant therapy were available for 12 patients. For all women the following data were obtained: date of the surgery, date of the most recent examination or death and time and site of relapse. Control follow-up examination was performed every 1 - 12 months. All subjects underwent a thorough physical examination, general and biochemical blood tests including neoplastic markers (CA15-3, CEA). Radiological examinations (mammography, chest and bone X-rays, computerised tomography), ultrasound, as well as magnetic resonance imaging and scintigraphy of bones were performed when necessary. In some cases recurrence was confirmed by fine-needle aspiration cytology and/or histopathology. A mean follow-up was 54.1 months. Relapse was detected in 64 women. In 17 cases it was a loco-regional recurrence (local recurrence, carcinoma in the second breast, metastases to supraclavicular lymph nodes), in 27 cases visceral metastases (brain, lung, pleura, liver, adrenals). Bone metastases were found in the remaining 20 women. A 50-month relapse-free survival was observed in 71.7% of women. Ductal carcinoma was graded according to Bloom and Richardson [5]. Table 1 summarizes basic clinical and morphological data.

Immunohistochemistry

Slices 5μm thick were deparaffinised, immersed in citric buffer and heated with micro waves. Sections were incubated with monoclonal antibodies: anti CD31 (JC/70A clone, Dako, Denmark; dilution 1:20), MIB-1 (Dianova, Hamburg; dilution 1:30) anti human estrogen receptor (Dako, Denmark; dilution 1:50) for 60 minutes at room temperature, and streptavidin-biotin-peroxidase (Histostan-SP kit, Zymed Laboratory's, San Francisco) was used. The sections were rinsed and stained with hematoxylin.

Microvessel density was measured according to Weidner et al. [23]. In the specimen highlighted by CD31 antibody light microscopy under low magnification (40x) was used to define the so-called hot spots with the highest microvessel density. Analysis was performed on hot spot with the largest number of microvessels (capillaries and small venules). Even single endothelial cells were regarded as vessels if they could be isolated from the adjacent microvessels, tumour cells and connective tissue elements. Vascular lumen was not a prerequisite for measuring the vessel. Vessels lying in hyalinised tumour sites and non-neoplastic adjacent tissue were excluded from analysis. Measurements were made using a computerised image analyser Quantimet 600S (Leica, Great Britain) combined with a microscope (Zeiss Axiophot, Germany) by means of a camera (3CCD Color Camera GP US 502 E, Panasonic, Japan). The measurements were made per 200x field of 0.74mm^2 in diameter. The values were calculated per 1mm^2 and analysed statistically in this form. The following parameters were measured: microvessel count (MCV), microvessel area (MVA) and microvessel perimeter (MVP). Mircovessel area was the sum of endothelial surface areas and vascular lumen areas. MVA was expressed in μm^2 and MVP in μm.

Data on proliferative index and estrogen receptor expression were obtained from a computerised database of the Department of Pathomorphology in Szczecin. Proliferative index i.e. the percentage of cell nuclei showing the product of reaction with monoclonal antibody MIB-1 was measured in 1000 nuclei of breast cancer. Nuclei with MIB-1 reactivity were considered as Ki-67 positive. Sites with the highest proliferative activity were selected for analysis. All nuclei with reactivity against estrogen receptor protein were considered as estrogen-positive. Measurements were done using a computerised image analyser Quantimet 600S under magnification x 400.

Statistical analysis

Non-parametric Mann-Whitney test was used to test the relationship between angiogenesis parameters and relapse.

TABLE 2

Microvessel count, area and perimeter related to relapse in women with invasive ductal breast carcinoma (means and standard deviations)

Patients	n	All			n	LN–			n	LN+		
		MVC±SD	MVA±SD	MVP±SD		MVC±SD	MVA±SD	MVP±SD		MVC±SD	MVA±SD	MVP±SD
With relapse	64	99.4±32.1	11067.2 ± 4580.8	4595.5 ±1520.7	16	92.7±39.8	10549.9 ±4941.5	4320.8 ±1783.7	48	101.7±29.2	11239.7 ±4495.9	4687.0 ±1431.9
Without relapse	162	66.0±24.6	7414.6 ±4110.0	3041.7 ±1266.7	95	56.7±18.3	6278.0 ±2794.7	2621.3 ±887.0	67	79.3±26.5	9026.1 ±5058.3	3637.7 ±1476.1
p		<0.0001	<0.0001	<0.0001		0.0002	0.0002	0.0001		<0.0001	0.006	0.0001

Explanations for Tables 1 - 4: MVC - microvessel count per 1mm^2 of tumour tissue; MVA - microvessel area (μm^2) per 1mm^2 of tumour tissue; MVP - microvascular perimeter (μm) per 1mm^2 of tumour tissue; All - all patients; LN– - node-negative patients; LN+ - node-positive patients; NA - not studied; NS - non-significant

TABLE 3

Percentage of patients with 50-month relapse-free survival according to MVC, MVA and MVP

Parameters of angiogenesis	Percentage of patients with 50-month relapse-free survival								
	All (n=226)			LN– (n=111)			LN+ (n=115)		
	MVC	MVA	MVP	MVC	MVA	MVP	MVC	MVA	MVP
Microvessel count									
>69.48	53.1			62.5			49.4		
≤69.48	90.3			94.9			79.4		
Microvessel area									
>7174.68μm^2		54.9			70.7			45.8	
≤7174.68μm^2		88.5			94.3			79.1	
Microvessel perimeter									
>3109.16μm			54.0			66.7			48.1
≤3109.16μm			89.4			94.7			78.9
p	<0.0001	<0.0001	<0.0001	<0.0001	=0.0007	<0.0001	=0.0004	=0.004	=0.002

Kaplan-Meier survival curve was used to show the relationship between angiogenesis parameters, some clinical and morphological variables and relapse-free survival. Differences between the curves were tested using the log-rank test. Proportional hazard Cox regression analysis was used to examine the independence of prognostic factors. A $p<0.05$ was considered as statistically significant.

Results

Relapse-free survival (RFS) was considered as the period between the date of surgery and the date of first recurrence irrespective of the site. A group of 226 women with invasive ductal breast cancer with known follow-up, time and site of the recurrence was used for statistical analysis. In 64 women relapse was observed between 4.2 and 49.8 months, whereas the remaining women had survived for 50 to 85.2 months since the operation.

Primary tumours of patients with recurrences had significantly higher MVC, MVA and MVP as compared to tumours of patients without recurrences, both in the whole group and in the subgroups with and without axillary lymph node metastases (Table 2). In node-negative patients relapse did not occur if MVC was below 38.7.

Among the 64 patients with relapse the parameters of angiogenesis in the primary tumour were higher in women who died than in those who survived 50.8 - 85.1 months (MVC 108.9 vs. 91.1; p=0.03, MVA 12307 vs. 9973.3; p=0.04, MVP 5054.7 vs. 4190.2; p=0.04).

Angiogenesis parameters in 81 women with primary tumour below 20mm in diameter and histological grade II differed significantly depending on the presence or absence of relapse. In primary tumours of women with relapse MVC (93.8 vs. 62.8, p=0.0006), MVA (11055.4μm^2 vs. 6879.8μm^2, p=0.008) and MVP (4347.9μm vs. 2835.7μm, p=0.002) were significantly higher than in women without relapse.

In 47 women with axillary lymph node negative ductal breast cancer grade II, angiogenesis parameters in primary tumours differed significantly depending on the presence or absence of relapse. In primary tumours of women with relapse MVC (76.4 vs. 53.1, p=0.04), MVA (8347.1μm^2 vs. 5829.5μm^2, p=0.02) and MVP (3463.5μm vs. 2439.8μm, p=0.02) were significantly higher than in women without recurrence.

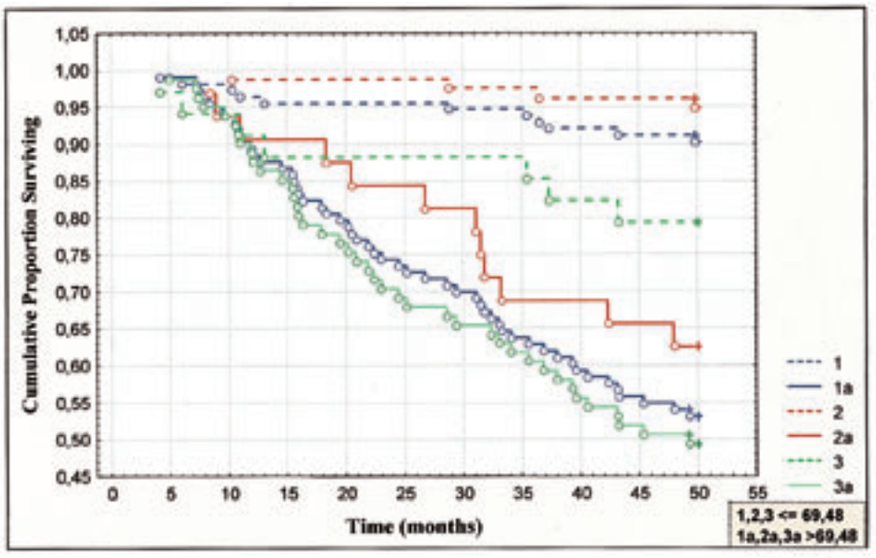

Fig. 1. The effect of microvessel count on relapse-free survival in the whole study population (1 vs. 1a), in the subgroup with node-negative disease (2 vs. 2a) and in the subgroup with node-positive disease (3 vs. 3a).

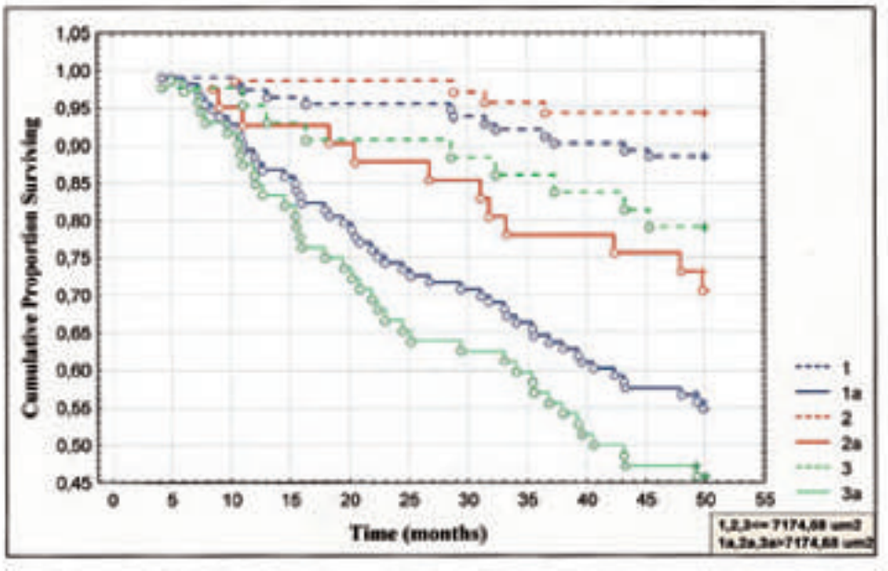

Fig. 2. The effect of microvessel area on relapse-free survival in the whole study population (1 vs. 1a), in the subgroup with node-negative disease (2 vs. 2a) and in the subgroup with node-positive disease (3 vs. 3a).

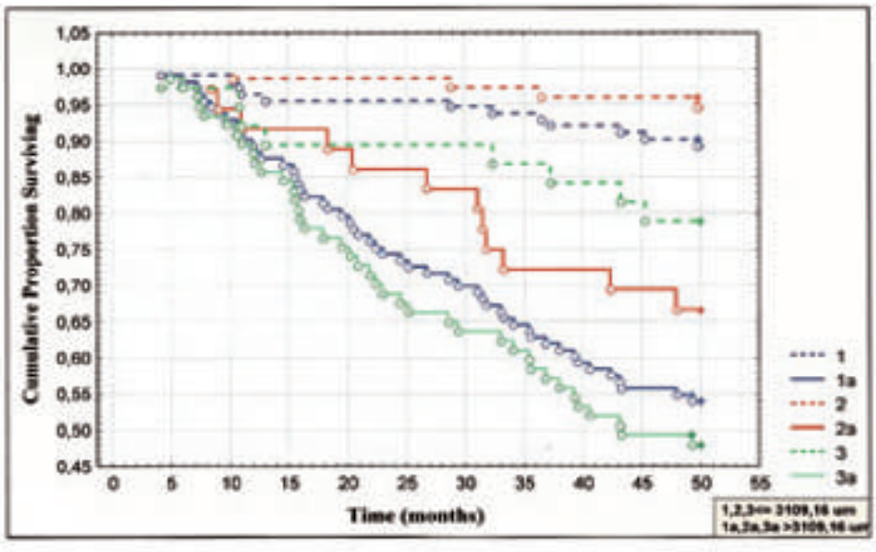

Fig. 3. The effect of microvessel perimeter on relapse-free survival in the whole study population (1 vs. 1a), in the subgroup with node-negative disease (2 vs. 2a) and in the subgroup with node-positive disease (3 vs. 3a).

TABLE 4

Multivariate Cox analysis with respect to 50-month relapse-free survival

Prognostic factor	Women with ductal breast carcinoma					
	All n=185		LN– n=94		LN+ n=91	
	HR	P	HR	P	HR	P
MVC	4.8	<0.0001	11.1	=0.0007	2.9	=0.02
Lymph nodes						
0+1–3 vs >3	4	<0.0001		NA		NA
1–3 vs >3	3.51	=0.0001		NA	3.51	=0.0001
ER	2	=0.03		NS		NS
Lymph nodes						
0+1–3 vs >3	4.4	<0.0001		NA		NA
1–3 vs > 3	3.36	=0.0004		NA	3.36	=0.0004
MVA	3.8	=0.0001	8.1	=0.0004	2.4	=0.03
ER	2	=0.03		NS		NS
MVP	4.5	<0.0001	9.7	=0.002	3	=0.009
Lymph nodes						
0+1–3 vs >3	3.9	<0.0001		NA		NA
1–3 vs >3	3.2	=0.0003		NA	3.2	=0.0003
ER	2	=0.02		NS	2	=0.04

For statistical analysis of the relationship between MVC, MVA and MVP in the primary tumour and the percentage of patients with 50-month RFS, ductal breast carcinomas were divided into two groups according to the median parameters of the intensity of angiogenesis. The median for MVC was 69.48, for MVA 7174.68μm^2 and for MVP 3109.16μm. Breast cancers with the parameters below or equal to the median were considered as low-MVC-, MVA- and MVP-carcinomas or carcinomas with low intensity of angiogenesis. Breast cancers with the parameters exceeding the median were considered as high-MVC-, MVA- and MVP-carcinomas or carcinomas with high intensity of angiogenesis.

There was a significant correlation between MVC, MVA and MVP and the percentage of patients with 50-month RFS (Table 3). Significantly fewer women with carcinomas with high intensity of angiogenesis survived 50 months without relapse as compared with women with carcinomas with low intensity of angiogenesis. This relationship was observed in all patients and in women with or without axillary lymph node metastases. Interestingly, in node-negative subgroup only 62.5% of women with tumors with high MVC and as much as 94.9% with low MVC survived 50 months without recurrence.

Kaplan-Meier survival curves indicate that high MVC (Fig. 1), MVA (Fig. 2) and MVP (Fig. 3) are associated with a lower probability of 50-month RFS as compared with patients having low MVC, MVA and MVP in the tumour. These relationships were observed in the whole group of patients (Fig. 1, 1 vs. 1a: $p<0.0001$; Fig. 2, 1 vs. 1a: $p<0.0001$; Fig. 3, 1 vs. 1a: $p<0.0001$), in node-negative subgroup (Fig. 1, 2 vs. 2a: $p<0.0001$; Fig. 2, 2 vs. 2a: $p=0.0007$; Fig. 3, 2 vs. 2a: $p<0.0001$) and in node-positive subgroup (Fig. 1, 3 vs. 3a: $p=0.004$; Fig. 2, 3 vs. 3a: $p=0.0004$; Fig. 3, 3 vs. 3a: $p=0.002$). For the node-negative subgroup MVC and MVP were the best discriminating parameters from the viewpoint of probability of relapse (the difference between the survival curves 32.4% - Fig. 1, 2 vs. 2a, and 28% - Fig. 3, 2 vs. 2a).

The relationship between age, tumour diameter, histological grade, axillary lymph node status and 50-month RFS was analysed in the group of 226 women with ductal breast carcinoma. The relationship between MIB-1 index and estrogen receptor expression in tumor cells, and 50-month RFS was studied in 192 and 189 cases, respectively. Relapse within 50 months after the operation was significantly affected by: (1) in the whole group: tumour diameter >20mm ($p=0.01$), lymph node metastases ($p<0.0001$) and no ER in cell nuclei ($p=0.02$); (2) in node-positive subgroup: the presence of more than three metastatic lymph nodes ($p=0.002$), high MIB-1 index ($p=0.03$) and no ER ($p=0.046$). In node-negative subgroup none of the parameters had a significant effect on 50-month RFS.

To find out which parameter is an independent prognostic factor we used Cox proportional hazard regression model to analyse MVC, MVA, MVP, age, tumour diameter, histological grade, lymph node status, MIB-1 index, ER expression. For statistical analysis only those cases were included in which all the above-mentioned parameters were analysed. As parameters of angiogenesis (MVC, MVA and MVP) are interrelated, they were tested separately by Cox model. Table 4 summarises the results of Cox analysis with respect to 50-month RFS, including only independent prognostic factors.

With respect to 50-month RFS, in the whole group: MVC, MVP, MVA, lymph node status and ER expression were found to be independent prognostic factors. High MVC and MVP were associated with a slightly higher risk of relapse than lymph node status. In node-negative subgroup

only MVC, MVP and MVA were the independent prognostic factors. In node-positive subgroup the following parameters were found to be independent prognostic factors: the number of positive lymph nodes, MVP, MVC and MVA. In this subgroup the presence of >3 lymph nodes with metastases was associated with a higher risk of relapse than high MVP, MVC and MVA. In node-positive subgroup ER expression appeared as an independent prognostic factor only when MVP was analysed.

Discussion

Axillary lymph node-negative patients with invasive ductal breast carcinoma generally have a good prognosis, nevertheless about 20 - 30% of them will have a relapse within 10 years. According to the outcomes of St. Gallen conference adjuvant chemotherapy is not recommended only in patients with node-negative cancers with low risk of relapse (positive ER expression, tumour diameter ≤20mm, histological grade I, age ≥35 years). These women can be treated with tamoxifen. Women with node-negative cancers but with a higher risk of relapse (positive ER expression, tumour diameter >20mm or histological grade II, III or age <35 years) should receive hormone therapy or hormonochemotherapy. Patients with ER-negative tumors should receive polychemotherapy [10].

The results of this study show that angiogenesis could be used to identify breast cancer patients at high risk of relapse. The mean parameters of angiogenesis were higher in invasive ductal breast carcinomas in women with relapse as compared with those without recurrence within 50 months after the operation. This correlation was observed in node-negative and node-positive subgroups of patients. In the node-negative subgroup there were no recurrences within 50 months after operation if MVC in the primary tumour was below 38.7. Furthermore, the significant relationship between the intensity of angiogenesis and death, which has been found in patients with relapse strongly argues in favour of the importance of using angiogenesis to assess the risk of early relapse. We also demonstrated that the intensity of angiogenesis is related to the risk of early relapse both in subgroup of women with grade II ductal breast carcinoma <20mm in diameter and in subgroup of women with grade II node-negative ductal breast carcinoma. For this reason angiogenesis could be used to stratify patients with grade II ductal breast carcinoma (a very heterogeneous group from the viewpoint of prognosis), to subgroups with good and poor early prognosis. The relationship between high MVC and shorter RFS in node-positive and node-negative cancers has already been reported in studies without computer-aided image analysis [1, 7]. However, the usefulness of the intensity of angiogenesis in assessment of the risk of early relapse in women with grade II node-negative breast cancer has not been studied so far. Only Karaiossifidi et al. [13] in a group of 52 women with grade II node-negative ductal breast carcinoma found that mean MVC in the tumour was higher in women with relapse or death (mean follow-up 9.5 years) as compared with relapse-free women. In their study anti Factor VIII antibody was used as an endothelial marker and MVC was measured according to Weidner et al. In the only study published to date on the relationship between angiogenesis and prognosis in ductal breast carcinoma with the use of anti CD31 antibody and computer-aided image analysis, Barbareschi et al. [3] found that high MVC, MVA and MVP were associated with shorter RFS. In their study in contrast to our results MVA has been a better prognostic parameter than MVP.

Multivariate Cox analysis revealed that MVC and MVP are the strongest independent prognostic factors with respect to RFS in the whole group of patients. In women with node-negative cancers MVC, MVP and MVA were the only independent prognosticators. In women with node-positive disease, although MVC, MVA and MVP were also the independent prognostic factors, the presence of more than 3 metastatic lymph nodes was associated with a higher risk of relapse than high vascular parameters. In other studies on ductal breast carcinoma, angiogenesis has also been shown to be the independent prognostic factor in multivariate analysis [1, 3, 7, 12]. Cox analysis in women with node-negative cancer has been performed only by Barbareschi et al. [3]. They found out that MVC and MVA but not MVP are the independent prognostic factors with respect to RFS. Only Axelsson et al. [2] have not found the relationship between angiogenesis and RFS in women with ductal breast carcinoma including those with node-negative disease. The following factors could be responsible for the differences: (1) in their series of node-negative cases two thirds constituted T2 and T3 tumors; (2) difficulties in identifying "hot spots"; (3) measurement of MVC in the field area smaller than that recommended by Weidner et al.

In our study 94.9% of women with node-negative breast cancer with low intensity of angiogenesis survived 50 months without relapse, whereas the proportion of corresponding women with high intensity of angiogenesis was 62.5%. Barbareschi et al. [3] obtained similar results (94% vs. 64%) as well as Chu et al. [7] (93% vs. 57%) at 50-month follow-up. The outcomes of St. Gallen conference suggest [10] that before treating breast cancers patients with low risk of relapse (positive ER expression, tumour diameter ≤20mm, histological grade I, age ≥35 years) the risk of side-effects of adjuvant therapy with tamoxifen should be considered versus potential advantages of decreased risk of relapse. We showed that angiogenesis is an independent prognostic factor indicating early relapse in node-negative subgroup of patients. Therefore we suggest that angiogenesis should be added to factors identifying low-risk node-nega-

tive breast cancer patients. This would facilitate better delineation of a subgroup with a minimal risk of relapse, which might not require treatment with tamoxifen. The results of our study imply that node-negative patients with low angiogenesis in the tumour are not candidates for standard adjuvant treatment since almost 95% of them will experience 50-month RFS.

In summary, computer-aided image analysis is a good and objective technique for evaluating the intensity of angiogenesis in breast cancer. The results of our study show that in women with node-negative and node-positive invasive ductal breast carcinoma the parameters of angiogenesis (MVC, MVP and MVA) in the primary tumour are independent indicators of the probability of early relapse. Furthermore MVC and MVP are comparable and at the same time better than MVA vascular parameters for the evaluation of the relationship between angiogenesis and RFS, which means they may be used alternatively. In women with node-negative disease particularly those with grade II tumors the evaluation of angiogenesis can identify a subgroup of patients with high risk of early relapse, hence it may help in decisions concerning adjuvant therapy. Patients with high angiogenesis could be the candidates for antiangiogenic treatment irrespective of other factors defining the risk of relapse. The combination of chemotherapy and antiangiogenic agents [6] seems especially advantageous. Further prospective studies are necessary to determine the prognostic value of assessment of parameters of angiogenesis by computerised image analysis especially in women with node-negative breast cancer.

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