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Prognostic value of intratumoral microvessel density, a measure of tumor angiogenesis, in node-negative breast carcinoma — results of a multiparametric study

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Summary

In the present study we update previous results on the prognostic value of intratumoral microvessel density (IMD), determined immunocytochemically using the monoclonal antibody CD-31 and a standard streptavidin-immunoperoxidase technique, published in the *J Clin Oncol* 12:454-466, 1994. This study was undertaken in those 211 node-negative breast cancer (NNBC) cases of that series of which we had pathological material available to determine all the prognostic indicators. The median period of follow-up has been extended to 78 and 80 months for relapse-free survival (RFS) and overall survival (OS), respectively, and new biological indicators (i.e. Ki-67 labeling and 67 kDa laminin receptor expression) were included in the analysis.

The main results obtained are: **i)** a confirmation that IMD is not associated with the other biological markers studied, i.e. expression of p53 protein, c-erbB-2 protein, 67 kDa laminin receptor, and cell kinetics; IMD was weakly associated only with histological grade ($p=0.053$); **ii)** IMD remains a highly significant prognostic factor for RFS and OS ($p<0.0001$ and $p=0.018$, respectively) in univariate analysis; **iii)** in multivariate analysis on RFS, IMD (likelihood ratio test (LRT)=30.16; $p<0.0001$), 67 kDa laminin receptor (LRT=9.80; $p=0.0017$), the IMD/67 kDa laminin receptor interaction (LRT=8.62; $p=0.0033$), tumor size (LRT=8.56; $p=0.0034$), and p53 protein (LRT=4.96; $p=0.025$) are significant and independent prognostic indicators. For OS, only tumor size (LRT=8.34; $p=0.0038$), menopausal status (LRT=5.16; $p=0.023$), p53 protein (LRT=4.37; $p=0.036$), and IMD (LRT=4.05; $p=0.044$) retain a significant and independent prognostic value.

The results of this study confirm the prognostic importance on RFS of the variables previously tested, but not of peritumoral lymphatic vessel invasion. A novel finding is that 67 kDa laminin receptor and the IMD/67 kDa laminin receptor interaction are also significant and independent variables. For OS, the results confirm that both IMD and tumor size are significant and independent variables. With prolonged

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follow-up the novel finding that emerges is the prognostic importance of menopausal status and p53 protein.

This new information could be useful for a more accurate selection of high-risk NNBC patients who require careful follow-up and may benefit from adjuvant therapy.

Introduction

Angiogenesis is a highly controlled process in normal tissues of the adult, being regulated by angiogenic peptides and natural angiogenesis inhibitors. Physiologically the inhibitory pathway predominates [1]. In contrast, solid tumors may stimulate formation of new vessels in the stroma, and there is growing evidence that both tumor growth and metastasis are angiogenesis-dependent [2,3]. During tumor progression the switch from the avascular to the vascular phase may be the result of decreased production of natural inhibitors [4] or of increased secretion of angiogenic factors [5,6]. This phenomenon is regulated by complex cellular [7-9], biochemical [10-11], and genetic [4,12-14] mechanisms.

Furthermore, endothelial cells may secrete growth factors and cytokines that stimulate growth of neoplastic cells by a paracrine system [15]. Thus, a reciprocal stimulation may operate between stromal intratumoral endothelial cells and the tumor parenchymal cells.

There is mounting evidence that angiogenesis plays a relevant role in the processes of transformation and progression of solid tumors. In fact, the acquisition of the angiogenic property is generally accompanied by enhanced tumor growth, progression, and metastasis in some experimental models [16-18].

Based on this biological background, several studies have evaluated the clinical significance of the determination of tumor angiogenesis in breast cancer [19] as well as in other solid tumors [20, 21], and most of the authors found that intratumoral microvessel density (IMD) is associated with metastasis and clinical outcome.

The aim of the present study is to update our previous study published in the *Journal of Clinical Oncology* [22] by: 1) adding to the determination of IMD novel biological markers, to study their relationship and prognostic value in a multivariate statistical analysis; and 2) extending the median period of observation of the series up to 6.5 years.

Patients and methods

The eligibility criteria for the inclusion of node-negative breast cancer patients, the surgical therapy, and the postoperative follow-up schedule were the same as those previously reported [22]. For the purpose of this study we evaluated only those 211 cases for which we had sufficient pathologic material to determine all the biological markers. The main pathologic characteristics of this series are shown in Table 1.

Immunocytochemical determinations

IMD

Intratumoral microvessels were identified by immunostaining in formalin-fixed, paraffin-embedded sections with anti-CD31 monoclonal antibody (clone JC/70A; Dakopatts A/S, Glostrup, Denmark) in a 1:200 dilution and incubating overnight [22]. H&E-stained sections were used to select areas representative of the invasive tumor component, and IMD was determined as previously described by Weidner et al [23]. Briefly, in the area of most intense neovascularization (hot spot), individual microvessel counts were first made on a 400 x field. Any brown-staining endothelial cell clearly separate from adjacent microvessels, tumor cells, and other connective-tissue elements was considered a single, countable microvessel. Immunocytochem-

Table 1. Association of IMD with the other pathobiological variables tested

Variable	N°of tumors	IMD median (mean)	IMD range	K[a]	p value
Histotype				0.60	0.85
ductal	165	84 (89.2)	15-222		
lobular and others	46	80 (90.5)	8-244		
Tumor size				0.57	0.89
pT1	132	80 (89.2)	20-244		
pT2-3	79	84 (89.9)	8-220		
Grading				1.34	0.053
GI-II	148	79 (85.0)	15-244		
GIII	63	90 (99.9)	8-220		
PLVI				0.79	0.55
–	186	83 (90.7)	8-244		
+	25	72 (80.5)	28-220		
ER-ICA[b]				0.67	0.74
+/++	155	80 (87.5)	8-244		
–	50	87 (93.5)	23-220		
PgR-ICA[b]				0.76	0.60
+/++	125	82 (89.5)	8-224		
–	80	80 (88.0)	15-220		
c-erbB-2				0.52	0.94
–	160	83 (89.0)	8-244		
+	51	80 (90.9)	24-222		
p53[c]				1.13	0.15
–	156	80 (87.9)	15-244		
+/++	54	91 (95.3)	8-215		

[a] by the Kolmogorov-Smirnov test; [b] data available for 205 cases; [c] data available for 210 cases.

ical stains for microvessels were performed by P.B. at the Institute of Pathology of St Bortolo Regional Medical Centre, Vicenza, by P.D.P. at the Institute of Pathology of St Chiara Regional Medical Centre, Trento, and by N.W. at the Department of Pathology, University of California, San Francisco. All of the immunostained slides were evaluated and the microvessels counted in a 0.74-mm^2 area (i.e. 200 x field) at the University of California, San Francisco, by N.W. Approximately 5 to 10 minutes is required to perform the microvessel count. While counting microvessels, N.W. had no knowledge of the results of the other prognostic factors and/or clinical outcomes. For the purpose of this study, IMD was assessed as a continuous variable.

p53 protein

Staining for the p53 protein was performed using the monoclonal antibody anti-p53 pAb 1801 (Oncogene Science Inc, Uniondale, NY), using a previously published method [22]. Briefly, formalin-fixed, paraffin-embedded 5-μm sections were rehydrated and incubated overnight at 4°C with the primary antibody diluted to 1:200. Biotinylated antimouse IgG (1:100) and a complex of streptavidin and biotinylated peroxidase (1:100) were added in sequence, followed by 3,3'-diaminobenzidine tetrahydrochloride development. Negative controls consisted of omission of the primary antibody. Positive controls were sections of squamous cell carcinomas known to have p53 gene mutations and p53 protein accumulation. Staining was evaluated by a semiquantitative

method as follows: (–) = negative, (+) = heterogeneous and moderate nuclear staining (<10%), and (++)= strong and diffuse nuclear labeling (>10%).

c-erbB-2 protein

This was determined on 5-μm sections of routine formalin-fixed, paraffin-embedded blocks, using the polyclonal antibody 21N and the immunocytochemical method previously published [22]. Positive controls included breast carcinomas known to exhibit high levels of protein. Negative controls were obtained by omission of the primary antibody. For the purpose of this study and according to the criteria of Wright et al [24], only individual tumors with membrane staining in at least 50% of the tumor cells were reported as positive. Diffuse cytoplasmic staining was ignored.

67 kDa laminin receptor (LRec)

The monoclonal antibody MLuC5 directed to the 67 kDa LRec was used [25]. The immunoperoxidase test was carried out on formalin-fixed, paraffin-embedded sections with the use of purified MLuC5 (10 μg/ml) and the avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, California) using the same method adopted by Martignone et al [26]. One 5-μm section for each carcinoma was examined, and the presence of LRec was estimated by counting the number of labeled cancer cells and was expressed as a percentage of the total number of cancer cells examined (at least 1,000 cells per case) by 2 independent observers.

Control slides were prepared by using preimmune rabbit immunoglobulins instead of the specific antibody.

Cell kinetics by the Ki-67 antibody

This was assessed in 5μm frozen sections of 105 tumors using the Ki-67 monoclonal antibody developed by Gerdes et al [27] purchased from Dako, Ltd, Glostrup, Denmark. The primary antibody was diluted 1:50 for 60 minutes and then tissue sections were incubated with avidin-biotinylated horseradish peroxidase complex (Vector Lab, Burlingame, CA) for 30 minutes as previously reported [28].

ER and PgR expression

ERs were analyzed using the monoclonal antireceptor antibody H-222 Sp2γ with a kit purchased from Abbott Diagnostic (Abbott Labs, North Chicago, IL). Sections were analyzed for PgR using the rat monoclonal antireceptor antibody immunoglobulin G (IgG) KD-68 with a kit assay purchased from Abbott Diagnostics.

For both assays, a negative control was established by replacing the primary antibody with normal rat IgG. As positive controls, a known ER- and PgR-positive specimen was incubated with the sections to be evaluated. At least 500 cells were counted from each tumor. Tumors were classified ER- or PgR-positive if at least 10% of cells showed nuclear staining and the results quantitated (1+ = heterogeneous and moderate staining with <30% of cells positive; 2+ = strong staining and >30% cells positive).

Statistical analysis

The relationship between IMD and the other continuous variables (LRec and Ki-67 labeling) was analyzed by means of the Spearman's coefficient. The distribution of IMD within the modalities of each of the other variables considered as dichotomous was compared by the Kolmogorov-Smirnov test. Since Ki-67 determination was done in only 105 tumors, this variable was not considered in survival analysis.

The patterns of OS and RFS were estimated by means of the product limit method (Kaplan-Meier) on the basis of a 6-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 evaluated using a log-logistic regression model where each regression coefficient is the log of the odds ratio (OR) and is constant in time [29]. IMD and LRec were analyzed as continuous vari-

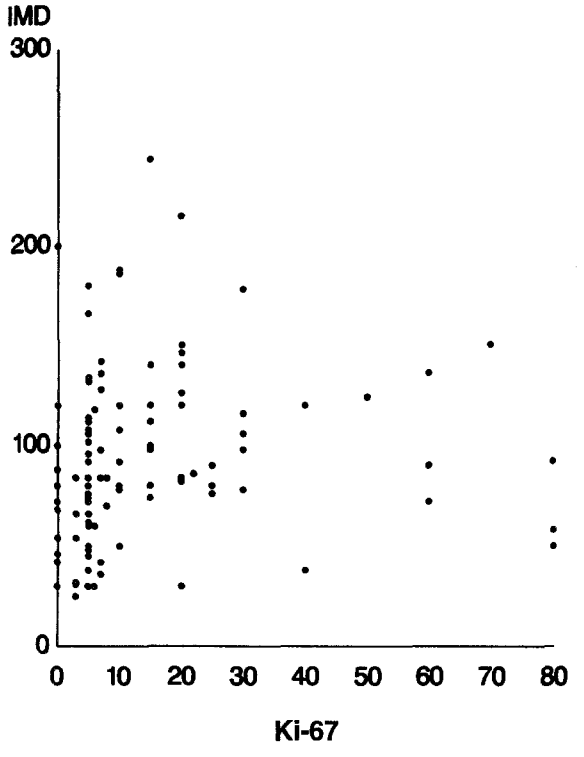

Figure 1. Lack of association between IMD and Ki-67 in 105 evaluable tumors. Both variables are considered continuous (Spearman's coefficient = 0.320).

ables. The use of a continuous variable (in its original measurement scale) in a log-logistic regression model requires a log-linear relationship between the variable and OR. The assumption of log-linearity was investigated with the method of Andersen et al [30]. For OS odds are the probability of dying over the probability of surviving; for RFS odds are the probability of relapsing over the probability of remaining disease-free. In the multivariate analysis are considered all those variables with OR significantly different from 1.0 in the univariate analysis, and the interaction terms considered biologically relevant (IMD and LRec, IMD and ER, and IMD and PgR). In univariate and multivariate analysis, for each regression coefficient, the null hypothesis $\beta=0$ (OR=1) was tested by Wald statistics. Starting with an initial model containing all the variables mentioned above, a final more parsimonious model was obtained using a backward selection procedure. In this procedure the contribution of each variable was evaluated by the likelihood ratio test (LRT). In the final model are considered only those variables with LRT p values<0.05.

Since the variables are not independent the results of univariate and multivariate analysis could be different. For this reason all the variables that were not statistically significant in univariate analysis were singly added in the final regression model to avoid neglecting an important prognostic factor.

In univariate and multivariate analysis for each variable, we considered the reference category to be that with the putative better prognosis.

Statistical analysis was performed using the Statistical Analysis System package (SAS Institute, Cary, NC).

Results

Intratumoral Microvessel Density (IMD) and its association with the other indicators

The distribution of the IMD has already been reported [22]. The present study, which includes novel variables such as the expression of the LRec and the determination of cell kinetics (done only in 105 cancers), confirms that IMD is not significantly associated with the other biological markers [22] (Table 1).

In particular, there was a poor linear relationship between IMD and Ki-67 (Spearman's coefficient = 0.320) (Figure 1), and between IMD and LRec (Spearman's coefficient = 0.144) (Figure 2). The only significant association observed was between IMD and histologic grade (Kolmogorov-Smirnov K'=1.34; p=0.053). In fact, GIII tumors had higher values of IMD than those GI-II.

Univariate analysis

The median follow-up of the patients has been

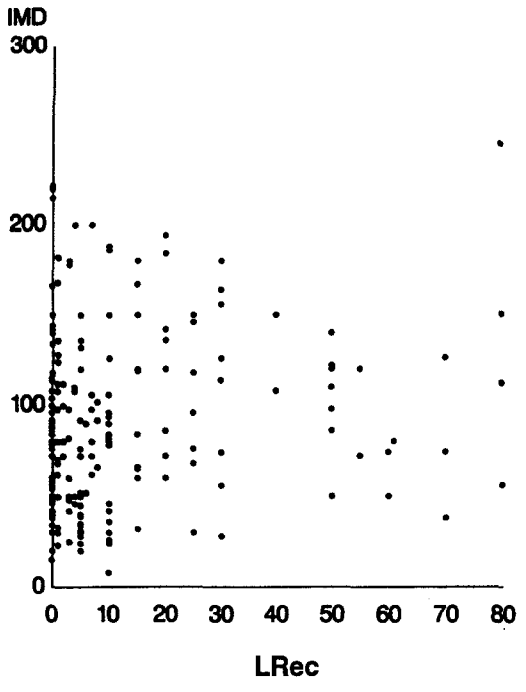

Figure 2. Lack of association between IMD and 67 kDa laminin receptor in 211 evaluable tumors. Both variables are considered continuous (Spearman's coefficient = 0.144).

extended to 78 and 80 months for RFS and OS, respectively. The probability of RFS and OS of this series of NNBC at 6 years was 74% and 84%, respectively. During the period of observation 31 patients died, 21 due to tumor progression and 10 due to causes other than breast cancer. 53 cases developed a recurrence.

This study with prolonged follow-up confirms that IMD is a significant prognostic indicator for both RFS and OS when evaluated as a continuous variable ($p<0.0001$ and $p=0.018$, respectively) (Figure 3a,b) as well as a dichotomous variable (data not shown). The other variables found to be statistically significant for RFS were: tumor size ($p=0.0019$), p53 protein expression ($p=0.0033$), and PgR status ($p=0.033$). Moreover, LRec expression approached significance ($p=0.072$) (Table 2).

As far as OS is concerned, p53 protein expression ($p=0.00099$), tumor size ($p=0.0045$), and menopausal status ($p=0.038$) were significantly prognostic. Again, LRec approached significance ($p=0.093$) (Table 3).

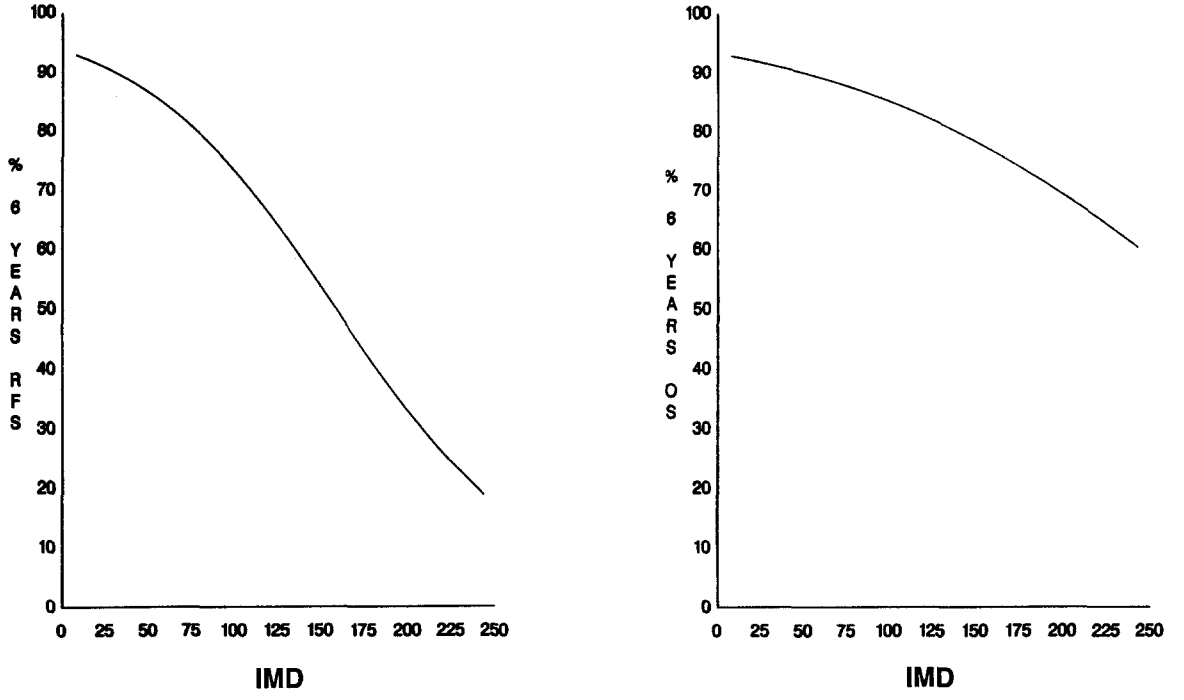

Figure 3. (a) 6-year RFS according to IMD as a continuous variable (number of microvessels per 0.74 mm^2 or 200X microscopic field), $p<0.0001$; (b) 6-year OS according to IMD as a continuous variable, $p=0.018$.

Table 2. Univariate analysis of RFS at 6 years

Variable	OR	95% CI	χ^2	p value
Menopausal status				
post vs pre/peri*	0.77930	0.3965 – 1.5128	0.5661	0.45180
Histotype				
ductal vs lobular & others*	0.63566	0.2766 – 1.4446	1.2142	0.27050
Tumor size				
pT1 vs pT2–3*	2.80571	1.4902 – 5.4834	9.5950	**0.00195**
Grading				
GI–II vs GIII*	1.58998	0.8157 – 3.1594	1.8979	0.16832
PLVI				
neg vs pos*	1.86194	0.7957 – 4.4236	2.1031	0.14700
ER–ICA				
+/++ vs –*	1.79873	0.8877 – 3.7523	2.6875	0.10114
PgR–ICA				
+/++ vs –*	2.00323	1.0568 – 3.9007	4.4985	**0.03392**
IMD				
continuous variable	1.01734	1.0107 – 1.0247	22.6803	**<0.0001**
c–erbB–2				
neg vs pos*	1.82039	0.9071 – 3.7210	2.8761	0.08990
p53				
– vs +/++*	2.76020	1.4240 – 5.5814	8.6134	**0.00334**
Laminin receptor				
continuous variable	1.01557	0.9985 – 1.0334	3.2340	0.07213

Abbreviations: OR=odds ratio; CI=confidence interval; *higher risk category

Since Ki-67 labeling results were available only in half of the series, this variable was excluded from survival analyses.

Multivariate analysis

In the initial model we considered all the variables with OR significantly different from 1.00 in univariate analysis, plus the following interaction terms: IMD and LRec, IMD and ER, IMD and PgR. The results of the final model are reported in Table 4.

For RFS, the interaction term between IMD and LRec was statistically significant ($p<0.01$). OR for the interaction term was less than 1.00. However, in this regression model the variables act in a multiplicative way on the odds and the joint prognostic effect of IMD and LRec was similar to that obtained considering the products of the single effects of IMD and LRec. For example, if we consider an increment of 10 vessels in IMD and of 10 percent in LRec, the OR is 2.436, whereas when the interaction term between the two variables is also allowed the resulting OR value is 2.315. So, although statistically significant, the interaction term between IMD and LRec does not seem to add relevant prognostic information.

For RFS, both IMD (LRT, $\chi^2=30.16$; $p<0.0001$) and LRec (LRT, $\chi^2=9.80$; $p=0.0017$) were statistically significant. The other prognostic factors were, in order of statistical significance, tumor size (LRT, $\chi^2=8.56$, $p=0.0034$) and p53 (LRT, $\chi^2=4.96$; $p=0.025$).

For OS, tumor size was the most significant independent variable in predicting death (LRT, $\chi^2=8.34$, $p=0.0038$). The odds of death were 3.13

Table 3. Univariate analysis of OS at 6 years

Variable	OR	95% CI	χ^2	p value
Menopausal status				
post vs pre/peri*	0.37682	0.1461 – 0.9430	4.2981	**0.03816**
Histotype				
ductal vs lobular and others*	1.53415	0.6521 – 3.6469	1.0338	0.30926
Tumor size				
pT1 vs pT2–3*	3.26660	1.5023 – 7.4066	8.0622	**0.00452**
Grading				
GI–II vs GIII*	1.35232	0.6026 – 3.0713	0.5811	0.44588
PLVI				
neg vs pos*	1.28883	0.4280 – 3.9076	0.2239	0.63604
ER–ICA				
+/++ vs –*	1.66890	0.7239 – 3.9302	1.5297	0.21615
PgR–ICA				
+/++ vs –*	1.40491	0.6437 – 3.1333	0.7839	0.37595
IMD				
continuous variable	1.00919	1.0017 – 1.0171	5.5624	**0.01835**
c–erbB–2				
neg vs pos*	0.99511	0.4010 – 2.4753	0.0001	0.99112
p53				
– vs +/++	2.86527	1.3194 – 6.5395	6.6510	**0.00099**
LRec				
continuous variable	1.01627	0.9970 – 1.0363	2.8623	0.09332

Abbreviations: OR=odds ratio; CI=confidence interval; *higher risk category

times higher in those patients with pT2-3 tumors than in those with pT1 tumors. The other most significant independent prognostic factors were, in order of statistical significance: menopausal status (LRT, χ^2=5.16, p=0.023); p53 protein expression (LRT, χ^2=4.37, p=0.036) and IMD (LRT, χ^2= 4.05, p=0.044) (Table 4).

Discussion

A marker of angiogenesis meets the first requirement of McGuire's guidelines for new prognosticators in early-stage breast cancer, namely a clear biological role in the processes of transformation, progression, and/or metastasis [31]. About 20 years ago, Gimbrone and Gullino [32] first demonstrated, using an experimental model of chemically induced mammary papillary hyperplasia of ductal origin, that papillomas serially transplanted may transform to mammary carcinomas after having acquired angiogenic activity. Subsequently, this observation was reproduced in human pathology [33], and Jensen et al [34] found that normal lobules from patients with invasive ductal carcinoma have a significantly higher angiogenic activity than those from non cancerous mammary glands. More recently, Weidner et al [23] and Guidi et al [35] found that a percentage of *in situ* ductal carcinomas have a pattern of vascularization. They suggested that the *in situ* lesions associated with higher angiogenesis may be those at higher risk of progression to invasive carcinomas.

Experimental studies performed in some human breast cancer cell lines have demonstrated that different angiogenic peptides play a role in transformation [36,37], progression [38], and

Table 4. Multivariate analysis of RFS and OS using a log-logistic regression analysis (final model)

Variable	OR	95% CI	Wald statistic		LRT	
			χ^2	p value	χ^2	p value
Recurrence-free survival						
IMD continuous variable	1.02373	1.0152–1.0334	25.9049	<0.0001	30.1611	**<0.0001**
p53 – vs +/++*	2.35634	1.1169–5.1818	5.0099	0.02520	4.9656	**0.02585**
LRec continuous variable	1.06777	1.0212–1.1191	7.9837	0.004720	9.8009	**0.001744**
IMD/LRec interaction	0.99949	0.9990–0.9999	5.9505	0.01471	8.6246	**0.003317**
Tumor size pT1 vs pT2-3*	2.82499	1.4151–5.9677	8.3855	0.003782	8.5645	**0.003428**
Overall survival						
IMD continuous variable	1.00801	1.0000–1.0163	3.8535	0.04964	4.0509	**0.04414**
p53 – vs +/++*	2.35440	1.0460–5.5272	4.2415	0.03947	4.3771	**0.03642**
Tumor size pT1 vs pT2-3*	3.13008	1.4030–7.3562	7.2127	0.00723	8.3431	**0.003871**
Menopausal status post vs pre/peri*	0.36662	0.1369–0.9489	4.2350	0.03959	5.1657	**0.02303**

Abbreviations: OR=odds ratio; CI=confidence interval; LRT=likelihood ratio test; *higher risk category

metastasis [39] of this neoplasm. In an elegant study, Zajchowski et al [40] observed that overexpression of thrombospondin, a potent natural angiogenesis inhibitor, suppresses the tumorigenic capability of somatic hybrid cells obtained by fusion of the MCF-7 human breast cancer line with immortalized human normal mammary epithelial cells. Thus, angiogenesis is necessary for breast cancer progression and, as observed in other solid tumors, the switch from the avascular to the vascular phase seems to be an early event, generally accompanied by rapid primary tumor growth and metastasis [16-18].

Weidner et al [23] first demonstrated the potential clinical usefulness of the determination of angiogenic activity, measured by counting intratumoral microvessels using a specific marker for vascular endothelial cells (factor VIII-related antigen), in a small series of breast cancer patients. As recently reviewed [19], from 1992 to 1994 13 subsequent clinico-pathological independent retrospective studies correlating vascular index and prognosis of patients with operable breast carcinoma were published. Overall, 2396 cases were evaluated, 1814 in 11 "pilot" and 582 in 2 "confirmatory" studies. The terms "pilot" and "confirmatory" prognostic study follow the guidelines recently proposed by Gasparini et al [41]. The majority of the authors confirmed a significant association between vascularization and axillary lymph nodes and/or distant metastasis, and they found that IMD is a significant prognostic indicator for RFS (8 of 12 studies) and OS (6 of 8 studies) [19]. Furthermore, another three studies [42-44], which evaluated angiogenic activity using biochemical methods by measuring

intratumoral concentrations of plasminogen activator inhibitor-1 (PAI-1), demonstrated that high levels of PAI-1 were associated with poor prognosis in breast cancer. Similarly, two studies performed by the Folkman group found that levels of basic fibroblast growth factor, a potent angiogenic peptide, in serum [45] or urine [46] were associated with clinical outcome and the "status" of the disease.

Overall, even if the optimum method to assess angiogenic activity in human pathology has not yet been identified, several clinical studies have confirmed experimental findings that elevated angiogenesis is associated with tumor biological aggressiveness and that it is a marker of poor clinical outcome.

The present study, performed in a series of NNBC patients with a median follow-up of 6.5 years, gives more details on the biological and clinical significance of the determination of intratumoral vascularization in node-negative breast cancer. First of all, we confirm [22,47] that IMD is not associated with the other pathobiological markers of emerging importance in breast cancer such as p53 protein, c-erbB-2 protein, 67 kDa laminin receptor, hormone receptors, and cell kinetics parameters [22,47,48].

These results are consistent with those obtained in our first study performed in another series of 165 stage I-II operable breast cancers [49-51], and with our biological knowledge on the role of the other endocrine, autocrine, and genetic factors implicated in the multi-step processes of transformation and progression of human breast cancer [52]. Secondly, the results of the univariate analysis on RFS and OS at 6 years demonstrated that the magnitude of the prognostic value of the determination of tumor angiogenesis remains similar to that found after 5 years [22].

Last but not least, the results of the multivariate analysis showed that IMD is the strongest significant and most independent prognosticator for RFS in a statistical model including other 4 variables, and that it retains an independent prognostic value for OS also when p53 protein, tumor size, and menopausal status are considered in the statistical model.

Besides these confirmatory results on the prognostic value of IMD, we found that the determination of the expression of p53 protein, 67 kDa laminin receptor, and tumor size adds independent prognostic information for RFS, and that p53 protein expression, tumor size, and menopausal status add independent prognostic information for OS.

As far as the prognostic contribution of the expression of 67 kDa laminin receptor is concerned, we have reported elsewhere that the co-determination of IMD and of this marker enhanced the prediction of clinical outcome in a series of 171 NNBC, particularly among those patients with low vascularized carcinomas [53].

However, for PLVI the prognostic value [22] was not confirmed in the present study. 25 tumors were PLVI-positive out of a total of 233 (10.7%) in the study previously reported [22] and 25 out of 211 assessable cancers (11.8%) in the present study. It is unlikely that the small difference in the percentage of PLVI-positivity between the two studies could be responsible for the different prognostic result observed for this factor. In our series, the prognostic significance of PLVI on RFS seems to decrease when the period of follow-up observation is extended.

In conclusion, this multiparametric study reporting the prognostic value of some pathobiological markers in a series of NNBC patients with a sufficiently long period of observation gives evaluable information useful for identifying those patients at high risk. This study should not be considered as conclusive, but it could constitute the basis for the choice of those markers which merit re-evaluation in a future larger multicentric study using a sophisticated neural network statistical analysis and involving a sufficiently large number of cases. This needs to be planned to obtain a reliable identification of those prognosticators which are effectively able to stratify subgroups of NNBC patients at different risk for whom different postsurgical therapeutic strategies may be indicated.

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References

1. Folkman J: How is blood vessel growth regulated in normal and neoplastic tissue? Cancer Res 46:467-475, 1986.
2. Folkman J: Introduction: Angiogenesis and cancer. Sem Cancer Biol 3:47-48, 1992.
3. Liotta L, Kleinerman J, Saldel G: Quantitative relationship of intravascular tumor cells, tumor vessels, and pulmonary metastases following tumor implantation. Cancer Res 34:997-1004, 1974.
4. Dameron KM, Volpert OV, Tainsky MA, Bouck N: Control of angiogenesis in fibroblasts by p53 regulation of thrombospondin-1. Science 265:1582-1585, 1994.
5. Millauer B, Shawver LK, Plate KH, Risau W, Ullrich A: Glioblastoma growth inhibited *in vivo* by a dominant-negative FIK-1 mutant. Nature 367:576-579, 1994.
6. Kandel J, Bossy-Wetzel E, Radvanyi F, Klagsbrun M, Folkman J, Hanahan D: Neovascularization is associated with a switch to the export of bFGF in the multistep development of fibrosarcoma. Cell 66:1095-1104, 1991.
7. D'Amore PA: Capillary growth: a two-cell system [review]. Sem Cancer Biol 3:49-56, 1992.
8. Leibovich SJ, Polverini PJ, Shepard HM: Macrophage-induced angiogenesis mediated by tumour necrosis factor-alpha. Nature 329:630-634, 1987.
9. Starkey JR, Crowle PK, Taubenberger S: Mast-cell-deficient W/W^v mice exhibit a decrease rate of tumor angiogenesis. Int J Cancer 42:48-52, 1988.
10. Goto F, Goto K, Weindel F, Folkman J: Synergistic effects of vascular endothelial growth factor and basic fibroblast growth factor in the bovine capillary endothelial cells within collagen gels. Lab Invest 69:508-517, 1993.
11. Brooks PC, Clark RAF, Cheresh DA: Requirement of vascular integrin $\alpha_V\beta_3$ for angiogenesis. Science 264: 569-571, 1994.
12. Rastinejad F, Polverini PJ, Bouk NP: Regulation of the activity of a new inhibitor of angiogenesis by a cancer suppressor gene. Cell 56:345-355, 1989.
13. Kieser A, Weich HA, Brandner G, Marmè D, Kolch W: Mutant p53 potentiates protein kinase C induction of vascular endothelial growth factor expression. Oncogene 9:963-969, 1994.
14. Bouck NP: Tumor angiogenesis: The role of oncogenes and tumor suppressor genes. Cancer Cell 2:179-185, 1990.
15. Rak JW, Hegman E, Lu C, Kerbel RS: Progressive loss of sensitivity to endothelium-derived growth inhibitors expressed by human melanoma cells during disease progression. J Cell Physiol 159:245-255, 1994.
16. Folkman J, Watson K, Ingber D, Hanahan D: Induction of angiogenesis during the transition from hyperplasia to neoplasia. Nature 339:58-61, 1989.
17. Fidler IJ, Ellis LM: The implications of angiogenesis for the biology and therapy of cancer metastasis. Cell 79:185-188, 1994.
18. O'Reilly MS, Holmgren L, Shing Y, Chen C, Rosenthal RA, Moses M, Lane WS, Cao Y, Sage EH, Folkman J: Angiostatin: A novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. Cell 79:315-328, 1994.
19. Gasparini G, Harris AL: Clinical importance of the determination of tumor angiogenesis in breast carcinoma: Much more than a new prognostic tool [review]. J Clin Oncol 13:765-782, 1995.
20. Craft PS, Harris AL: Clinical prognostic significance of tumour angiogenesis [review]. Ann Oncol 5:305-311, 1994.
21. Gasparini G: Quantification of intratumoral vascularisation predicts metastasis in human invasive solid tumours [review]. Oncol Rep 1:7-12, 1994.
22. Gasparini G, Weidner N, Bevilacqua P, Maluta S, Dalla Palma P, Caffo O, Barbareschi M, Boracchi P, Marubini E, Pozza F: Tumor microvessel density, p53 expression, tumor size, and peritumoral lymphatic vessel invasion are relevant prognostic markers in node-negative breast carcinoma. J Clin Oncol 12:454-466, 1994.
23. Weidner N, Semple SP, Welch WR, Folkman J: Tumor angiogenesis and metastasis — correlation in invasive breast carcinoma. N Engl J Med 324:1-8, 1991.
24. Wright C, Angus B, Nicholson S, Sainsbury JRC, Cairns J, Gullick WJ, Kelly P, Harris AL, Horne CHW: Expression of c-erbB-2 oncoprotein: A prognostic indicator in human breast cancer. Cancer Res 49:2087-2090, 1989.
25. Martignone S, Pellegrini R, Villa E, Tandon NN, Mastroianni A, Tagliabue E, Ménard S, Colnaghi MI:

Characterization of two monoclonal antibodies directed against the 67KDa high affinity laminin receptor and application for the study of breast carcinoma progression. Clin Exp Metast 10:379-386, 1992.

26. Martignone S, Ménard S, Bufalino R, Cascinelli N, Pellegrini R, Tagliabue E, Andreola S, Rilke F, Colnaghi MI: Prognostic significance of the 67-kilodalton LRec expression in human breast carcinomas. J Natl Cancer Inst 85:398-402, 1993.
27. Gerdes J, Schwab U, Lamke H, Stein H: Production of mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. Int J Cancer 31:13-20, 1983.
28. Gasparini G, Pozza F, Bevilacqua P, Meli S, Boracchi P, Reitano M, Santini G, Marubini E, Sainsbury JRC: Growth fraction (Ki-67 antibody) determination in early-stage breast carcinoma: Histologic, clinical, and prognostic correlations. The Breast 1:92-99, 1992.
29. Gore SM, Pocock SJ, Kerr GR: Regression models and non-proportional hazards in the analysis of breast cancer survival. Appl Statist 33:176-195, 1984.
30. Andersen PK, Borgan O, Dill RD, Keiding N (eds): Statistical Models Based on Counting Processes. Springer-Verlag, New York, 1993.
31. McGuire WL: Breast cancer prognostic factors: Evaluation guidelines [editorial]. J Natl Cancer Inst 83:154-155, 1991.
32. Gimbrone MA Jr, Gullino PM: Neovascularization induced by intraocular xenografts of normal, preneoplastic, and neoplastic mouse mammary tissues. J Natl Cancer Inst 56:305-318, 1976.
33. Brem SS, Jensen HM, Gullino PM: Angiogenesis as a marker of preneoplastic lesions of the human breast. Cancer 41:239-244, 1978.
34. Jensen HM, Chen I, DeVault MR, Lewis AE: Angiogenesis induced by "normal" human breast tissue: A probable marker for precancer. Science 218:293-295, 1982.
35. Guidi AJ, Fischer L, Harris JR, Schnitt SJ: Microvessel density and distribution in ductal carcinoma in situ of the breast. J Natl Cancer Inst 86:614-619, 1994.
36. Arteaga CL, Carty-Dugger T, Moses HL, Hurd SD, Pietempol JA: Transforming growth factor-beta(1) can induce estrogen-independent tumorigenicity of human breast-cancer cells in athymic mice. Cell Growth Differ 4:193-201, 1993.
37. Costa M, Danesi R, Agen C, Di Paolo A, Basolo F, Del Bianchi S, Del Tacca M: MCF-10A cells infected with the *int*-2 oncogene induce angiogenesis in the chick chorioallantoic membrane and in the rat mesentery. Cancer Res 54:9-11, 1994.
38. Kurebayashi J, McLeskey SW, Johnson MD, Lippman ME, Dickson RB, Kern FG: Quantitative demonstration of spontaneous metastasis by MCF-7 human breast cancer cells cotransfected with fibroblast growth factor-IV and lacZ. Cancer Res 53:2178-2187, 1993.
39. McLeskey SW, Kurebayashi J, Honig SF, Zwiebel J, Lippman ME, Dickson RB, Kern FG: Fibroblast growth factor-IV transfection of MCF-7 cells produces cell lines that are tumorigenic and metastatic in ovariectomized or tamoxifen-treated athymic nude mice. Cancer Res 53:2168-2177, 1993.
40. Zajchowski DA, Band V, Trask DK, Kling D, Connolly JL, Sager R: Suppression of tumor-forming ability and related traits in MCF-7 human breast cancer cells by fusion with immortal mammary epithelial cells. Proc Natl Acad Sci USA 87:2314-2318, 1990.
41. Gasparini G, Pozza F, Harris AL: Evaluating the potential usefulness of new prognostic and predictive indicators in node-negative breast cancer patients [review]. J Natl Cancer Inst 85:1206-1219, 1993.
42. Grondahl-Hansen J, Christensen IJ, Rosenquist C, Brunner N, Mouridsen HT, Dano K, Blichert-Toft M: High levels of urokinase-type plasminogen activator and its inhibitor PAI-1 in cytosol extracts of breast carcinomas are associated with poor prognosis. Cancer Res 53:2513-2521, 1993.
43. Janicke F, Schmitt M, Graeff H: Clinical relevance of the urokinase-type and tissue-type plasminogen activators and of their type 1 inhibitor in breast cancer. Sem Thromb Hemostasis 17:303-332, 1991.
44. Foekens JA, Schmitt M, vanPutten WLJ, Peters HA, Kramer MD, Janicke F, Klijn JGM: Plasminogen activator inhibitor-1 and prognosis in primary breast cancer. J Clin Oncol 12:1648-1658, 1994.
45. Li V, Yu C, Rupnick M, Allred E, Sallan S, Hayes DF, Folkman J: Serum from breast cancer patients contains proliferative activity for capillary endothelial cells which correlates with risk of mortality [abstract #252]. Proc Am Soc Clin Oncol 12:113, 1993.
46. Nguyen M, Watanabe, Budson AE, Richie JP, Hayes DF, Folkman J: Elevated levels of an angiogenic peptide, basic fibroblast growth factor, in the urine of patients with a wide spectrum of cancers. J Natl Cancer Inst 86:356-361, 1994.
47. Horak ER, Leek R, Klenk N, Le Jeune S, Smith K, Stuard N, Greenall M, Stepniewska K, Harris AL: Angiogenesis, assessed by platelet/endothelial cell adhesion molecule antibodies, as indicator of node metastases and survival in breast cancer. Lancet 340: 1120-1124, 1992.
48. Fox SB, Gatter KC, Bicknell R, Going JJ, Stanton P, Cooke TG, Harris AL: Relationship of endothelial cell proliferation to tumor vascularity in human breast cancer. Cancer Res 53:4161-4163, 1993.
49. Weidner N, Folkman J, Pozza F, Bevilacqua P, Allred EN, Moore DH, Meli S, Gasparini G: Tumor angio-

genesis: A new significant and independent prognostic indicator in early-stage breast carcinoma. J Natl Cancer Inst 84:1875-1887, 1992.

50. Bevilacqua P, Boracchi P, Gasparini G: Prognostic indicators for early-stage breast carcinoma. Part II: Value of cathepsin D expression, detected by immunocytochemistry. A multiparametric study. Int J Oncol 5:559-564, 1994.
51. Weidner N, Gasparini G: Determination of epidermal growth factor receptor provides additional prognostic information to measuring tumor angiogenesis in breast carcinoma patients. Breast Cancer Res Treat 29:97-107, 1994.
52. Marx J: Cellular changes on the route to metastasis. Science 259:626-629, 1993.
53. Gasparini G, Barbareschi M, Boracchi P, Bevilacqua P, Verderio P, Dalla Palma P, Menard S: 67 kDa laminin receptor expression adds prognostic information to intra-tumoral microvessel density in node-negative breast cancer. Int J Cancer 60:604-610, 1995.