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Clinical Significance of the Determination of Angiogenesis in Human Breast Cancer: Update of the Biological Background and Overview of the Vicenza Studies

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INTRODUCTION

HUMAN INVASIVE breast cancer is a malignancy characterised by a complex biology that influences the tumour–host relationship and responsiveness to anticancer treatments [1]. A better understanding of the most important biological pathways regulating transformation, progression and metastasis of this tumour is a prerequisite for the development of more rational, specific and effective treatments and for improving the management of patients. Currently, we know that angiogenesis is necessary for the growth of solid tumours and that acquisition of the angiogenic phenotype facilitates local invasiveness, progression and the development of distant metastasis [2].

Mounting data now indicate that there is both indirect and direct evidence that breast cancer is an angiogenesis-dependent disease. The first study of Weidner and associates [3] published in January 1991 in the *New England Journal of Medicine* suggested a methodological approach to assess angiogenic activity in human breast cancer, with potentially important prognostic applications. This study [3] stimulated several authors to perform independent studies worldwide to assess the prognosis of the patients and to improve the methodology.

To date, at least 21 clinicopathological studies reporting on the prognostic value of angiogenesis have been published in peer reviewed journals (Table 1) [4–24] and several new studies continue to be presented at international meetings. Thus, among the biological prognostic indicators, the assessment of angiogenesis is one of the more studied and it is one of the more promising.

This paper will review the biological role of angiogenesis in breast cancer, the methods used to determine angiogenic activity and the main results of our studies as well as of those from other institutions on the prognostic value of intratumoral microvessel density. Finally, some perspectives for future studies will be suggested.

BIOLOGICAL BACKGROUND

Angiogenesis (neovascularisation) is the process which permits a tumour to stimulate the formation of new blood vessels from the pre-existing vascular bed. The mechanisms leading to acquisition of the angiogenic phenotype are only partially known and involve complex biochemical, molecular, genetic and cellular mechanisms. Ultimately, the angiogenic activity of a tumour is the result of the equilibrium between angiogenic and angiosuppressive pathways [25].

In general, angiogenesis facilitates tumour growth, progression and metastasis through three main mechanisms: (i) the perfusion effect—neovascularisation supplies an appropriate delivery of oxygen and nutrients from blood circulation necessary for tumour growth; (ii) the metastatic effect—the formation and growth of intratumoral neovessels is accompanied by structural and morphological alterations that facilitate the penetration and transport of tumour cells in the bloodstream that colonise distant tissues; and (iii) the mutual paracrine effect—tumour cells produce growth factors able to stimulate angiogenesis, but endothelial cells also secrete some cytokines (e.g. IL-6, granulocyte colony-stimulating factor) and growth factors (e.g. angiogenic peptides and others), that directly stimulate tumour cells to grow (reviewed in [26,27]).

Indirect evidence that breast carcinoma is angiogenesis-dependent: experimental studies

Approximately 20 years ago, Brem and associates [28] developed an ocular model to assess angiogenic activity. They studied the angiogenic response induced in the rabbit iris by the transplantation of xenografts of normal, hyperplastic and chemically-induced neoplastic mouse mammary fragments. They found that angiogenesis preceded the transformation of mammary hyperplasia to carcinoma [28]. Further studies performed by Jensen and coworkers [29], using a similar experimental model, showed that angiogenesis is involved in human breast cancer transformation and progression. The angiogenic activity of normal breast lobules from cancerous breasts was twice that of non-cancerous breasts. Even though these studies gave stimulating results, no relevant further research was performed on angiogenesis in breast cancer during the 1980s. Only in the 1990s did independent experimental studies add new important information in this field. Studies by McLesky and associates [30] and Costa and associates [31] demonstrated that fibroblastic growth factors (FGFs)-3 and -4 induce angiogenesis followed by rapid tumour growth and metastasis in human breast cancer cell lines. Zhang and colleagues [32] found that human breast carcinoma cells transfected

Table 1. Intratumoral microvessel density and prognosis in operable breast carcinoma

Authors	Endothelial marker	Number of patients	Stage	Number of node-negative patients	Association with RFS	Association with OS	Multivariate analysis RFS	Multivariate analysis OS	Median follow-up (years)
Pilot studies									
Horak and associates [4]	CD-31	103	I–II	64	ND	0.006	ND	ND	2.5
Weidner and associates [5]	fVIII-RA	165	I–II	83	<0.001	<0.001	<0.001	<0.001	4.0
Bosari and associates [6]	fVIII-RA	180	I–II	151	<0.004	<0.008	<0.03	<0.03	9.0
Visscher and associates [7]	Type IV collagenase	58	I–IV	28	0.001	ND	NS	ND	5.1
Van Hoef and associates [8]	fVIII-RA	93	I	93	NS	NS	NS	NS	13.0
Khanuia and associates [9]	fVIII-RA	164	I–II	125	NS	ND	ND	ND	8.0
Obermair and associates [10]	fVIII-RA	64	I–II	32	<0.01	ND	<0.01	ND	4.1
Ogawa and associates [11]	fVIII-RA	155	I–II	91	<0.001	0.025	<0.002	<0.001	7.0
Hall and associates [12]	fVIII-RA	87	I–II	50	NS	ND	NS	ND	Cohort 1 = 9.5 Cohort 2 = 1.5
Fox and associates [13]	CD-31	211	I–II	112	ND	0.02	ND	0.05	3.5
Costello and associates [14]	fVIII-RA	87	I	87	NS	NS	ND	ND	2.9
Goulding and associates [15]	CD-34	165	I–II	ND	NS	NS	ND	ND	12.0
Siitonen and associates [16]	fVIII-RA/ CD-31/CD-34	77	I	77	ND	ND	ND	ND	8.0
Toi and associates [17]	fVIII-RA/ CD-31	125	I–II	57	<0.01	ND	<0.01	ND	5.1
Gasparini and associates [18]	CD-31	191	II	0	<0.01	<0.01	<0.01	<0.01	5.5
Macaulay and associates [19]	CD-31	88	I–II	23	0.022	ND	ND	ND	2.5
Confirmatory studies									
Bevilacqua and associates [20]	CD-31	211	I	211	<0.0001	0.018	<0.0001	0.044	6.6
Toi and associates [21]	fVIII-RA	328	I–II	130	<0.001	ND	<0.0001	ND	4.6
Obermair and associates [22]	fVIII-RA	230	I	320	<0.0001	<0.0001	ND	<0.001	4.6
Axelsson and associates [23]	fVIII-RA	220	I–II	110	NS	NS	NS	NS	11.5
Gasparini and associates [24]	CD-31	178	II	0	<0.01	<0.01	<0.01	<0.01	5.2

ND, not done; NS, not significant; RFS, relapse-free survival; OS, overall survival.
Note: When the same author performed more than one pilot or confirmatory study using the same method, only the most recent or most representative is reported.

with vascular endothelial growth factor (VEGF) exhibit rich vascularity and enhanced growth.

Overall, the results of these studies are in agreement with those generally observed in solid tumours, that different angiogenic peptides induce angiogenesis and facilitate progression and metastasis *in vivo*.

Other experimental studies have shown that the expression of endogenous angiogenesis inhibitors, such as thrombospondin-1 (TSP-1) [33–35] or transforming growth factor β-1 [36], are capable of inhibiting tumour growth and metastasis of human breast cancer cell lines *in vivo*, through the inhibition of neovascularisation. Of particular biological importance was the demonstration that one of the mechanisms of control of TSP-1 is mediated by the wild-type *TP53* tumour-suppressor gene [34].

Indirect evidence that breast carcinoma is angiogenesis-dependent: clinical studies

Brem and associates [37] in 1978 using a rabbit iris assay, showed for the first time that angiogenesis is a potentially useful marker of preneoplastic lesions and that it is associated with most human breast cancers. An iris angiogenic response was observed in 30% of fragments from biopsies of human hyperplastic lobules of the breast versus 65% of those from invasive breast carcinomas. Moreover, they observed that the frequency of transformation was higher in the subgroup of hyperplastic lesions with angiogenic activity. Weidner and associates in 1991 [30], examining histological sections of breast cancer stained with a specific marker for endothelial cells, showed that it is possible to observe *in situ* carcinomas in the prevascular as well as in the vascular phase. They suggested that the carcinomas *in situ* with angiogenic activity are potentially those with greater risk of progressing to invasive tumours. A subsequent study of Guidi and colleagues [38] gave more information on the histological patterns of stromal neovascularisation of *in situ* carcinomas of the breast.

Another histopathological study by Guinebretiere and coworkers [39] suggested that the risk of women with fibrocystic disease developing invasive breast carcinoma increases with the density of microvessels within the lesion. Independent studies published in 1995 indicate that some integrins, namely $\alpha_v\beta_3$ (vitronectin), $\alpha_v\beta_5$ and α_6, modulate angiogenesis and are involved in breast cancer progression [40–42]. Hildenbrand and associates [43] found that plasminogen activators are also involved in breast cancer neo-

vascularisation. Urokinase plasminogen activator (uPA) is produced by both cancer cells and macrophages and exerts a paracrine effect on endothelial cell growth. In a series of breast cancers, a significant association among uPA levels, high macrophage content and intratumoral microvessel density was observed [43]. An elegant study by Contrino and colleagues [44] suggested that the *in situ* detection of tissue factor seems to be a useful marker of angiogenic phenotype of human breast disease. The results of several clinicopathological studies have confirmed experimental observation of the presence in breast cancer of more angiogenic peptides which stimulate angiogenesis and progression.

It has been shown that human breast cancer expresses diverse angiogenic peptides including VEGF, FGFs, platelet-derived growth factor (PDGF) and the hepatocyte growth factor (HGF) [45–49], some of which are also detectable in the body fluids of patients [48, 50, 51]. Among these, VEGF seems to play a major role, and preliminary findings suggest that expression of VEGF is associated with high intratumoral microvessel density (IMD) [47] and prognosis [52].

An overview of the results of the numerous studies performed to study the correlation between vascularisation and prognosis suggests that human breast cancer presents a heterogeneous degree of angiogenic activity and that highly vascular tumours are biologically more aggressive and associated with poor outcome (Table 1).

Finally, the study of McCulloch and associates [53] showed that the extent of tumour cells shedding into effluent venous blood during breast cancer surgery are associated with the degree of vascularisation of the primary tumour, confirming preclinical studies indicating that angiogenesis facilitates metastases [27].

Direct evidence that breast carcinoma is angiogenesis-dependent: experimental studies

At least three published studies indicate that inhibition of angiogenesis is associated with human breast cancer regression in animal models. Weinstat-Saslow and colleagues [35] transfected TSP-1 complementary DNA into a metastatic human breast cancer cell line. This genetic modulation drastically inhibited angiogenesis *in vivo* and the treated animals exhibited reduced primary tumour growth and a reduction in the number of metastasis versus untreated controls. The second study from Brooks and associates [40] showed that systemic treatment with an antibody (LM-609) neutralising the $\alpha_v\beta_3$ integrin drastically reduced angiogenesis and induced tumour regression in a severe combined immunodeficient (SCID) mouse/human chimera bearing subcutaneous transplant of human breast cancer. In the third study, from Professor Folkman's group [54], angiostatin, a potent endogenous angiogenesis inhibitor, was administered to nude mice bearing diverse solid tumours, including the MDA–MBV human breast cancer. The systemic administration of angiostatin induced regression of all the transplanted human tumours through inhibition of angiogenesis and prolonged tumour dormancy via a mechanism mediated also by apoptosis.

Overall, when the results of these studies are considered together, there emerges a clear picture that angiogenesis is necessary (but not sufficient alone) for breast cancer growth, and that inhibition of angiogenesis is a promising new therapeutic approach (alone or in combination with other biological or conventional anticancer treatments) for breast cancer, worthy of clinical testing.

OVERVIEW OF THE VICENZA STUDIES

Our enthusiasm for translational research on angiogenesis was stimulated by the paper published by Weidner and associates [3] in 1991. The majority of our studies [5, 18, 20, 55, 56] have been in collaboration with Professor Noel Weidner using the method reported in the *New England Journal of Medicine* [3]. The steps and the recommendations for applying this method correctly have previously been detailed [57] and are also reviewed in this Special Issue of the *European Journal of Cancer* (pages 2506–2512).

Methodology

Weidner's method. The staining of the endothelial cells was achieved using the factor VIII-related antigen or the CD-31 antibody with a standard immunoperoxidase technique in paraffin sections. In all these series, the evaluation was performed by at least two observers and generally, after an adequate period of training on the counting technique, the correlation between counts on the same slide between different observers was satisfactory.

Computer-aided image analysis system (CIAS). In collaboration with the pathologists of the Trento Hospital, and the physicians of the Institute of Physics of the University of Trento, we developed a semi-automatic method to assess not only microvessel density, but also the total microvessel area and the vascular perimeter within the same field. We assessed the correlation between Weidner's method and the CIAS method and their prognostic value in a series of node-negative breast cancers [59]. We found a significant correlation between the subjective counts (Weidner's method) and those obtained by the CIAS, after which the same investigators chose the vascular hot spot to be used for the evaluations. The results of multivariate analysis indicated that both the subjective method and the total microvessel area calculated using the CIAS were significant prognostic indicators for relapse-free survival (RFS). The CIAS method seems to be potentially useful as a more objective method of quantifying tumour angiogenesis.

Chalkley score. Fox and associates [13] first proposed the Chalkley method, consisting of the use of a 25-point eyepiece graticule to be oriented so that the maximum number of points are on or within the stained vessels. The aim was to render the determination of microvessel density easier and objective. In collaboration with Professor Adrian L. Harris and his colleagues of the I.C.R.F. at the University of Oxford, we evaluated the Chalkley score and its prognostic value in a series of 178 patients from the two institutes with node-positive breast cancer treated with adjuvant tamoxifen [24]. Even though a higher Chalkley score was found in the Oxford series, the variable 'Centre' and its interaction with the Chalkley score were not statistically significant in the multivariate analysis, indicating that the prognostic value of this variable was similar in the two cohorts of patients. In this series, the degree of angiogenesis was not associated with any other variable and was significantly prognostic.

Novel methods. We are performing collaborative studies in order to evaluate new potentially useful techniques for

assessing angiogenesis for prognostic purposes. These include the intratumoral determination of the cytosolic concentrations of VEGF using an immunoenzymatic method, the assessment of the fraction of proliferating intratumoral endothelium using a double staining immunohistochemical technique to stain concurrently endothelial cells and proliferating nuclei, and the expression of the $\alpha_v\beta_3$ integrin. We are also testing the clinical significance of determining the levels of some angiogenic peptides in the serum of breast cancer patients.

The Vicenza clinical studies on the prognostic value of angiogenesis

We conducted several studies to evaluate the prognostic value of angiogenesis in breast cancer along the guidelines we proposed in 1993 [1]. To date, we have performed the pilot and confirmatory retrospective studies that will briefly be overviewed here.

Pilot studies. These were performed in an unselected and consecutive series of node-negative and node-positive breast cancer patients with a minimum follow-up of 4 years.

The first study was in collaboration with Professors Folkman and Weidner and was performed in a series of 165 patients (83 node-negative and 82 node-positive) [5]. We assessed both the conventional prognostic indicators (age, menopausal status, tumour size, histological grade, node status and peritumoral lymphatic vessel invasion) and several new biological indicators: epidermal growth factor receptor, c-erbB-2 expression, cathepsin-D, Ki-67 (a marker of cell kinetics), intratumoral microvessel density and grade using immunohistochemical methods as well as hormone receptors using standard biochemical assays and ploidy and S-phase fraction using flow cytometric analysis. We studied the association between angiogenesis and the other variables and its prognostic significance for RFS and overall survival (OS).

This study showed that (a) the degree of angiogenesis was significantly higher in node-positive tumours, larger primaries and poorly differentiated carcinomas; (b) angiogenesis was not associated with the other biological variables considered and (c) angiogenesis was a statistically significant prognostic indicator for both RFS and OS in the overall series and also separately in the subgroups of node-positive and node-negative patients, in multivariate analyses. In the same series, these results were confirmed in subsequent analyses with a median follow-up of 5 years [55] and also when we added to the multivariate model determination of the expression of p53 (pAb 1807 antibody) [56]. The encouraging results of these studies stimulated us to evaluate further the prognostic value of angiogenesis, assessed using the Weidner method, in larger and more homogeneous series.

Confirmatory studies in node-negative patients. To validate further the prognostic value of angiogenesis in this subgroup of patients, we assessed vascularisation in series treated only with surgery and with a median follow-up exceeding 5 years. The first confirmatory study was carried out in 254 unselected and consecutive patients operated in two Italian hospitals and clinically managed with similar protocols [60]. We assessed angiogenesis using the CD-31 antibody and also determined the expression of p53 and c-erbB-2. In this series, the degree of angiogenesis was significantly higher in premenopausal patients and in poorly differentiated tumours. No association was found between angiogenesis, p53, c-erbB-2 and hormone receptors. Multivariate analysis showed that angiogenesis, p53, tumour size and peritumoral lymphatic vessel invasion were statistically significant prognostic indicators for RFS, whilst for OS only angiogenesis and tumour size retained significance.

In the same series, we updated the results by extending the median follow-up to 6.5 years [20] and by adding to the analysis the determination of the 67 kDa laminin-receptor expression (MLuC5 antibody) [61]. These studies confirmed that angiogenesis is not associated with the other biological variables and that it is a significant prognostic factor for both RFS and OS, in a multivariate analysis model that included p53 and laminin receptor. A new finding was that with prolonged follow-up and with more events, menopausal status and p53 expression were also statistically significant prognostic indicators for OS [20].

Confirmatory studies in node-positive patients. The pilot studies [5, 55, 56] suggested that angiogenesis was an independent variable in the subgroup of node-positive breast cancer that included patients treated with heterogeneous forms of adjuvant therapy (either chemotherapy or hormone treatments). In order to study the relationship between angiogenesis and prognosis in relation to the adjuvant therapy administered, we performed another retrospective study in a series of 191 patients treated with adjuvant hormone therapy (84 cases) or with a standard chemotherapy (CMF schedule) (107 cases), followed for a median of 6 years [18]. We assessed angiogenesis using the CD-31 antibody and found that, irrespective of the adjuvant treatment administered, patients do worse as the degree of angiogenesis increases. On the basis of these results, we performed two confirmatory studies performed in larger series treated with homogeneous adjuvant treatments. In both these studies, we evaluated the prognostic value of angiogenesis using a new method based on the Chalkley score. In a series of 178 node-positive, tamoxifen-treated (20 mg daily for 3 years) patients, the median follow-up exceeding 5 years, we confirmed that angiogenesis is a significant prognostic indicator for both RFS and OS as are the number of involved nodes, grading, oestrogen receptor level in the tumour (only for RFS) and tumour size (only for OS) in multivariate analysis. This study was done in collaboration with Professor Harris's group [24].

The second collaborative study was performed in another series of 201 node-positive patients, median follow-up of 5.5 years, treated with adjuvant chemotherapy (CMF or CMF-like regimens) for 6 months (6–8 cycles). Again, multivariate analysis indicated that angiogenesis, number of involved nodes and progesterone receptor levels in the tumour were statistically significant prognostic indicators for both RFS and OS (G. Gasparini, unpublished study).

The most important, common finding of these confirmatory studies is that the patients with highly vascularised tumours had a significantly poorer outcome than those with poorly vascularised tumours, and that conventional adjuvant therapy seems unable to modify this relationship. It remains to be demonstrated in properly-designed prospective studies whether angiogenesis is merely a prognostic indicator or if it is predictive of efficacy of specific forms of adjuvant treatments in node-positive breast cancer.

Table 2. Prognostic value of intratumoral microvessel density in operable breast cancer. Data elaborated from the studies detailed in Table 1

	21 studies (3180 patients, 1754 node-negative)			
Univariate analyses				
	RFS = 18 studies		OS = 13 studies	
	Positive	Negative	Positive	Negative
	12 (67%)	6	9 (69%)	4
Multivariate analyses				
	RFS = 13 studies		OS = 10 studies	
	Positive	Negative	Positive	Negative
	9 (69%)	4	8 (80%)	2

RFS, relapse-free survival; OS, overall survival; positive, statistically significant association with prognosis; negative, lack of statistically significant association with prognosis.

Prognostic significance of the determination of intratumoral microvessel density (IMD) in operable breast cancer: overview of the literature

Table 1 shows the results of the most important published studies on vascularisation and prognosis in operable breast cancer. Overall, almost 3200 cases of breast cancer have been assessed for angiogenesis worldwide, and, as shown in Table 2, most have described a statistically significant association of IMD with prognosis. In particular, 69% and 80% of the studies, which included multivariate analyses, found that angiogenesis is a statistically significant prognostic indicator for RFS and OS, respectively.

Possible explanations for the results of some negative studies, relating to methodological problems, have been suggested elsewhere [57, 62].

Taking into account the heterogeneity of the clinicopathological characteristics and the treatments of the series analysed, that different markers and methods have been used to stain endothelia, and that no uniform criteria of evaluation were adopted, angiogenesis emerges as one of the most interesting biological indicators to assess the prognosis of breast cancer patients.

Because all the studies performed thus far are retrospective, a proper assessment of the clinical usefulness of the determination of IMD needs well-designed prospective, controlled, clinical trials in both node-negative and node-positive patients [1]. Finally, the clinicopathological studies on angiogenesis have also provided important collateral information on the characteristics of angiogenic activity in human breast cancer as summarised in Table 3 [4, 5, 20, 43, 53, 63–65].

ALTERNATIVE AND INVESTIGATIVE METHODS TO ASSESS ANGIOGENIC ACTIVITY

Some preliminary studies have evaluated the prognostic value of the determination of angiogenic peptides. The main results of these studies are summarised in Table 4 [45, 50, 52].

In collaboration with Dr Toi of the Metropolitan Hospital of Tokyo, we used an immuno-enzymatic technique to detect cytosolic concentrations of VEGF in 260 primary node-negative patients treated only with surgery. At a median follow-up of 5 years, we found that VEGF concentrations were significantly associated with the clinical outcome of the patients (G. Gasparini, unpublished study). These results are concordant with those previously observed where approximately 50% of breast cancers expressed this peptide and an association with prognosis was shown [52].

Recently, Contrino and associates [44] detected the *in situ* expression of the tissue factor, a regulator of coagulation, and found that its expression on vascular endothelium correlates with the malignant phenotype of human breast disease. The authors suggested that tissue factor is a potentially useful marker for identifying the switch to the angiogenic phenotype. Further studies in larger series are needed to evaluate the clinical significance of this marker.

Another new interesting marker for assessing angiogenesis in human breast cancer is the integrin $\alpha_v\beta_3$. Brooks and associates [40] found that elevated expression of this integrin is associated with active angiogenesis in human breast cancer, and that the blocking of its biological activity by neutralising antibodies causes breast cancer regression in animals.

Finally, several studies, recently overviewed by Duffy [66], indicate that determination of the plasminogen path-

Table 3. Collateral biological information from the clinicopathological studies on angiogenesis

Intratumoral microvessel density (IMD) is significantly associated with some angiogenic peptides [47, 52]
IMD is significantly associated with urokinase-type plasminogen activator (uPA), an enzyme involved in the degradation of extracellular matrix and angiogenesis [43]
IMD is not associated with other biological markers including p53, c-erbB-2, EGFR, tumour cell proliferation, cyclins, ploidy and hormone receptors [4, 5, 20]
IMD is independent of endothelial and tumoral cell proliferation [1, 65]
IMD is associated with tumour cell shedding into the bloodstream during surgery [53]

Table 4. Determination of angiogenic peptides and breast cancer prognosis

Author	Method	Number of patients	Stage	Number of node-negative patients	Association with therapy	Univariate analysis RFS	Univariate analysis OS	Multivariate analysis RFS	Multivariate analysis OS	Median follow-up (years)
PDGF										
Seymour and associates [45]	IHC	58	III–IV	ND	Yes	ND	ND	ND	ND	ND
VEGF										
Toi and associates [52]	IHC	103	I–II	30	ND	<0.01	ND	0.039	ND	4
Gasparini (unpublished results)	IE	260	I	260	ND	<0.01	<0.01	<0.01	<0.01	5
bFGF										
Nguyen and associates [50]	IE	143	I–IV	ND	ND	<0.05	NS	ND	ND	ND

PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor; bFGF, basic fibroblastic growth factor; IHC, immunohistochemistry; IE, immuno-enzymatic; ND, not done; NS, not significant.

ways is associated with breast cancer prognosis. Because modulation of plasminogen activator in tumour cells is involved both in invasiveness and angiogenesis, the related markers may be successfully used to assess some steps of angiogenesis in human solid tumours [43].

A more detailed description of new methods for the determination of angiogenesis, with an analysis of the advantages and disadvantages of each technique, has recently been published elsewhere [67].

CONCLUSION

There is sufficient evidence that the determination of IMD is a feasible quantitative static measure of angiogenic activity of breast cancer [57, 58, 62] and solid tumours in general [68].

As reported by Vermeulen and associates in this Special Issue of the *European Journal of Cancer* (pages 2474–2484), a consensus has been reached among several investigators to improve the immunohistochemical technique and to make the criteria for evaluating microvessel counts more objective and reproducible. Some validation procedures and quality control protocols have been recommended for future studies.

Angiogenesis is a multistep and complex phenomenon, so it seems reasonable to think that a more comprehensive and dynamic assessment of angiogenic activity will require the concurrent use of more assays and, in particular, the knowledge of the net balance between angiogenic stimuli and angiogenic inhibitors in each tumour [67].

In breast cancer, at least three areas of translational research on angiogenesis seem to be particularly important. First, preliminary studies on the angiogenic activity in benign [39], preneoplastic [37] and pre-invasive [38] breast diseases suggest that the acquisition of the vascular phenotype may appear at different times during the multistep processes leading to invasive carcinoma [69]. The search for markers capable of distinguishing the prevascular from the vascular phase of premalignant breast diseases may be a useful tool for identifying those women with breast lesions most likely to evolve into invasive cancer. Furthermore, such a marker could also be a target for chemoprevention using angiogenesis inhibitors to block the switch to the vascular phase and consequently inhibit local tumour invasiveness and progression [69, 70].

Second, approximately three-quarters of the retrospective studies published to date that evaluated the prognostic value of vascularisation in breast cancer found a significant association of high angiogenic activity with poor clinical outcome (Table 1). This relationship has been found in mixed, in node-negative as well as node-positive series of operable breast cancer. Definitive proof of the clinical significance of distinguishing high- versus low-risk patients by the angiogenic activity of their tumour, requires that this marker be evaluated in prospective controlled clinical trials in homogeneous groups of patients in relation to clinicopathological characteristics and therapy.

Third, once anti-angiogenic drugs are available for phase II–III clinical trials, it will be of great interest to verify in operable breast cancer whether the assessment of IMD or angiogenic peptides and endogenous angiogenesis suppres-

Table 5. Conclusions

Human invasive breast cancer is an angiogenic-dependent disease. Angiogenesis is necessary but not sufficient (alone) for breast cancer progression and metastasis
Determination of angiogenic activity is a promising prognostic indicator. Several methods to assess angiogenesis are available (IHC to detect IMD and angiogenic peptides, IE to detect angiogenic peptides, ELISA to detect plasminogen pathways, etc.). Prospective comparisons of different methods, standardisation and quality controls are warranted
Determination of angiogenesis seems to be useful for predicting the outcome of patients treated with adjuvant conventional therapy
Pharmacological and genetic modulation of angiogenesis is a highly effective and low toxic antitumour therapy in experimental models. A proper clinical development of anti-angiogenic drugs for therapy of breast cancer needs appropriate study designs and the assessment of biological effects using surrogate markers

IHC, immunohistochemistry; IMD, intratumoral microvessel density; IE, immuno-enzymatic.

sors may be targets for angiosuppressive therapy [71, 72]. Alternatively, future research should be planned to identify specific markers related to the modality of action of each angiogenesis inhibitor. For example, potential targets for drugs that inhibit proliferating endothelia (TNP-470, angiostatin, IL-12) may be the expression of markers associated with activated endothelium (antibodies TEC-11, E-9). Similarly, expression of the $\alpha_v\beta_3$ integrin may be a target for therapy with neutralising antibodies [40], or levels of tissue metalloproteinases may be related to responsiveness to metalloproteinase inhibitors (BB-94) [73]. Finally, the determination of tissue expression or serum/urine levels of angiogenic peptides may be useful for identifying the predominant pathway of activation of angiogenesis in each tumour that could be neutralised using antibodies or drugs against the specific endothelial growth factor [74, 75].

In conclusion, our present knowledge indicates that angiogenesis is involved in breast cancer progression and metastasis, and that its pharmacological inhibition is a promising new therapeutic approach. Well-designed studies of translational research on angiogenesis are warranted to define properly the assays for determining angiogenic activity, the prognostic and predictive value of angiogenesis activity, and to identify the patients who are most likely to benefit from pharmacological modulation of angiogenesis in general, or from specific anti-angiogenic drugs in particular (Table 5).

Anti-angiogenic agents are biological response modifiers, so it is likely that the key for a proper selection of the patients potentially responsive to them is the characterisation of the angiogenic activity of the tumour that is the target of their antitumoral effect [71].

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