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Review

Second international consensus on the methodology and criteria of evaluation of angiogenesis quantification in solid human tumours

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1. Introduction

During the 5 years since the publication of the first international consensus on the methodology and criteria of evaluation of angiogenesis quantification in solid tumours [1], new concepts and mechanisms of tumour vascularisation have been described with a major impact on the prognostic/predictive value of angiogenesis quantification and on the strategies of development of anti-angiogenic and vascular-targeting agents in oncology. The second consensus report aims at integrating these important novelties in updated guidelines for the estimation of the amount of blood vessels in a solid tumour and for the estimation of the ongoing angiogenesis. Qualitative aspects of tumour blood vessels will also be addressed, since these aspects are associated with a tumour vasculature established by mechanisms other than angiogenesis.

Redundancy appears to be characteristic to the development of vertebrates and of tumour growth, as demonstrated by several knock-out mice models. This is also illustrated by the alternative mechanisms solid tumours apply to obtain blood vessels. The statement of Judah Folkman that tumours need angiogenesis to grow, invade and metastasise [2] is no longer applicable to all tumours. Tumours, as all tissues and organs do, need blood vessels, but these blood vessels are not necessarily derived by sprouting of new blood vessels from pre-existing ones as in angiogenesis.

1.1. Alternative mechanisms of tumour vascularisation

In primary non-small cell lung carcinomas, an alveolar growth pattern was observed [3]. In this growth pattern, tumour cells filled the alveolar spaces, entrapping, but not destroying, the alveolar septa with the co-opted blood vessels. In the tumour cell nests, no associated desmoplastic stroma or new blood vessels were present. This putatively non-angiogenic growth pattern was also observed in lung metastases [4]. The lung tumours expressing this growth pattern were more often poorly differentiated than tumours expressing other growth patterns.

Holash and colleagues [5] have studied early vascularisation of gliomas in a rat model. Although the prevailing view was that malignancies and metastases initially reside as small avascular masses, 1 week after implantation of tumour cells, well-vascularised tumours were observed in their model. The tumour cells had co-opted the blood vessels of the surrounding brain tissue. There was no angiogenic response in the tumours. As a consequence of tumour growth in the absence of angio-

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genesis, the centre of the tumours became hypovascular. Later on, frank vessel regression led to tumour cell death. This tumour cell loss and hypoxia resulted in intense angiogenesis at the tumour margins. Autocrine expression of Angiopoietin-2, the endogenous Tie-2 receptor antagonist, marked the co-opted vessels of 2-week gliomas. At this stage, vascular endothelial growth factor (VEGF) expression in tumour cells was absent or low. After vessel regression and subsequent hypoxia, VEGF upregulation induced growth of the Angiopoietin-2 expressing and therefore destabilised blood vessels.

In this model of tumour vascularisation, co-option is restricted to the early phase of tumour growth. Compelling morphological evidence in human tumours suggests that co-option of pre-existing blood vessels might persist during the growth of primary tumours and of metastases, especially in well-vascularised organs such as the lungs and the liver. In liver metastases of colorectal adenocarcinomas, three growth patterns were observed [6]. In one of the patterns, tumour cells replaced the hepatocytes of the liver cell plates, while conserving the supportive tissue including the sinusoidal blood vessels. The endothelial cells of these co-opted sinusoids did not express the full spectrum of endothelial cell markers, which is comparable to the lack of expression of endothelial cell antigens on sinusoids in the normal adjacent liver parenchyma. The fraction of proliferating endothelial cells was small. Other investigators identified a subgroup of squamous cell lung carcinomas with low microvessel density and high tumour cell proliferation characterised by a high take rate in nude mice and by a short survival of patients [7].

A second alternative mechanism of tumour vascularisation, and a matter of vigorous debate [8], was described by Maniotis and colleagues [9] in aggressive human uveal and cutaneous melanomas. The tumour cells were found to line vascular channels forming a perfused network in the absence of endothelial cells, and expressing genes associated with vascular development, such as the endothelial receptor kinase Tie-1. In the mosaic blood vessels described by Chang and colleagues [10], the luminal surface is formed by both endothelial cells and tumour cells. Approximately 14% of the investigated blood vessels in human colorectal cancer biopsies showed mosaicism. The formation of mosaic vessels is distinct from the 'vasculogenic mimicry' described by Maniotis and colleagues [9] as the tumour cells of mosaic blood vessels do not show an endothelial phenotype.

Vasculogenesis, or the growth of blood vessels out of angioblasts, also seems to occur in tumour tissue. In a murine bone-marrow transplantation model, bone-marrow-derived Flk-1- or Tie-2-expressing endothelial cell progenitors or angioblasts were abundant in the highly vascularised peripheral regions of implanted colon cancer [11]. An increase in circulating endothelial cell progenitors was observed following VEGF administration in this model [12]. In patients with chronic myelogenous leukaemia carrying the *BCR/ABL* fusion gene, this gene was detected by *in-situ* hybridisation in endothelial cells generated *in vitro* from bone-marrow-derived progenitor cells and in vascular endothelium in myocardial tissue [13], corroborating the concept of vascular endothelium maintenance by bone-marrow-derived endothelial cells.

Intussusceptive vascular growth refers to vascular network formation by insertion of interstitial tissue columns into the vascular lumen and subsequent growth of these columns resulting in partitioning of the vessel lumen [14]. Endothelial cell division is not required for this form of vascular remodelling.

1.2. Quantification of angiogenesis or tumour vascularisation: prognostic/predictive value in oncology

The College of American Pathologists has investigated the prognostic and predictive factors in breast cancer and has stratified these into categories reflecting the strength of the published evidence [15]. Quantification of tumour angiogenesis by counting microvessels in immunostained tissue sections was ranked in category III, encompassing "all factors which are not sufficiently studied to demonstrate their prognostic value". The issues of methodological variation mentioned include: antibody selection, type of fixative used, methods of counting vessels, calculation of microvessel density, observer variability (especially of the selection of the field in which to count) and cut-off value for 'increased' vascularity. The current consensus report will address these issues and aims to lower the methodological variabilities of angiogenesis quantification in tumour tissue sections. New parameters measuring endothelial cell phenotypic characteristics of activation and differentiation and taking into account the angiogenesis-independent mechanisms of tumour vascularisation, will be presented.

2. Role of vascular differentiation and activation markers in tumour angiogenesis quantification (Table 1)

The stimulation of vascular endothelial proliferation results in upregulation of many biochemical pathways. Examples include upregulation of Kdr by VEGF [16], the urokinase receptor, CD105, which is a Transforming Growth Factor β (TGFβ)-receptor and Flt-4, which is the VEGF-receptor 3 [17,18]. The VEGF-receptor 3 is normally only expressed in lymphatics but in tumour vessels it becomes abnormally upregulated. The basement membrane is not properly formed, so there may be loss of differentiation markers as indicated by an epi-

Table 1
Important vascular differentiation and activation markers in tumour angiogenesis: study design and results *(antibody (clone))*

• *In vitro* upregulation of Kdr/Flk-1 receptor expression in endothelial cells by vascular endothelial growth factor (VEGF) [16]. (*moABs 3E7, GV39M*, *11B5*, *2C3 against the VEGF:Flk-1 complex* [31,37]).
• Analysis of the expression patterns of urokinase-plasminogen activator (u-PA) and uPA-receptor (r) in renal cell carcinomas: overexpression of uPA-r, u-PA and t-PA in capillaries within the tumour and tumour-associated macrophages [17]. (*moAb anti-uPA-r*: *R2 and R4*; *polyAb anti-uPA-r*; *moAb anti-uPA* (*MuK4, Biopool*), *moAb anti-tPA* (*anti-t-PA*, *Biopool*)).
• Microvessel density determined by anti-CD105 immunostaining in breast cancer: CD105 expression correlates with overall and disease-free survival [30]. (*moAb E-9*).
• Comparison of immunohistochemical expression of VEGF-receptor3-and its ligand VEGF-C in normal breast tissue and in breast carcinomas: increased number of VEGF-receptor3 positive vessels in breast cancer, of which part are blood vessels [18]. Comparable findings were reported for lung adenocarcinoma [38]. (*moAb 2E11 and 7B8 against the extracellular protein domain of the VEGF-receptor3*).
• Vascular maturation index assessed by staining with antibodies LH39 and anti-CD31 in non-small cell lung cancer: low angiogenesis and high maturation index were associated with better survival [19]. (*moAb LH39 against a non-collagenous component of the lamina lucida, a part of the basement membrane containing the anchoring filaments*).
• The ED-B domain of fibronectin, a 91 amino acid sequence, is inserted into the fibronectin molecule by alternative splicing, and accumulates around neovascular structures [29]. (*anti-ED-B single-chain Fv antibody fragments* (*L19*)).
• Vascular pericytes in human breast cancer are detected by an antibody against high molecular weight melanoma-associated antigen. Matrix Metalloproteinase (*MMP*)-*9* mRNA detection by *in situ* hybridisation and protein detection by immunohistochemistry show that pericytes produce MMP-9 in breast cancer [20]. (*moAb against HMW-MAA* (*clone NKI-M6*, *Monosan*)).
• Increased expression of E-selectin and vascular Cell Adhesion Molecule (VCAM-1) in endothelial cells of 64 invasive breast carcinomas, most prominent at the tumour periphery [22]. (*moAb against VCAM-1/CD106* (*clone 4B2*, *R&D Systems*), *and against E-selectin/CD62E* (*clone 1.2B6*)).
• Ligation of αvß3 integrin and of a growth factor receptor are necessary for sustained mitogen-activated protein kinase activity during angiogenesis [27]; vascular expression of αvß3 integrin in vascular hot spots predictive of relapse-free survival in 197 breast cancer patients [36] (*anti-αvβ3 integrin Ab* (*clone LM609*)).

tope detected by the antibody LH39 [19]. New fibronectin splice variants may be upregulated in the extracellular matrix and the pericytes and smooth muscle cells are greatly decreased. The latter may be detected by high molecular weight melanoma antigen and alpha smooth muscle actin antibodies, respectively [20].

Thus, in addition to counting the microvessels, analysis of their state of differentiation may give further information. Their composition can be better analysed, since in some tumours pericytes and smooth muscle cells contribute to angiogenesis [21]. This may be of value to select therapy—for example, tumours with high amounts of vascular VEGF may be more responsive to Kdr inhibitors or anti-Kdr antibodies, although it is currently not known how the phenotype affects response to therapy. More poorly differentiated vessels may provide easier access for cancer cells to the circulation, or may be more hypoxic because of an aberrant oxygen supply. The clinical significance is poorly studied, but could be analysed by applying such markers in prognostic studies.

Other markers reflect the effects of cytokines: E-selectin and Vascular Cell Adhesion Module (VCAM)-1 are often differentially upregulated in a heterogeneous pattern in tumours [22]. The endothelial proliferation index also varies markedly from tumour to tumour and is variably correlated with microvessel density [23–26]. αvβ3 Integrin is upregulated in most tumour endothelium [27], being an important target for therapy.

There are few studies that have compared these activation or differentiation markers with microvessel density and prognosis. Small studies have documented the heterogeneity, but not the significance. Often the marker does not correlate with microvessel density, e.g. fibronectin isoforms ED-A and ED-B [28], or LH39 [19]. Nevertheless, antibodies to these isoforms produced selective vascular targeting in tumours [29]. Some larger studies have shown that the detection of vessels expressing CD105 [30] and using antibodies to VEGF bound to Kdr [31] do give a better prognostic separation than microvessel density alone. In addition, loss of the LH39 epitope has shown [19,32] in 2 studies a relationship to regional node metastasis and thymidine phosphorylase expression. The association of poor differentiation with node metastasis may explain the variable correlation of microvessel density with this feature, with a more abnormal stroma and vessel structure favouring spread to the lymphatics.

Unlike normal blood vessels, vessels incorporated in solid tumours are structurally and functionally abnormal. The poorly differentiated vasculature is tortuous, dilated, and contains saccular blood vessels. These morphological characteristics have been measured in experimental settings. Regression of androgen-dependent tumours in a murine model was induced by castration which led to a decrease of VEGF expression. This VEGF withdrawal was followed by endothelial cell apoptosis and blood vessel regression before the

decrease in tumour size. The regressing blood vessels underwent normalisation. They became less tortuous and of a smaller diameter [33]. A beneficial effect of this would be an increased delivery of anti-tumour cell chemotherapeutic agents to the neoplasm. Therefore surrogate markers that permit the *in vivo* quantification of these phenotypical changes are needed in clinical trials that combine anti-angiogenesis with chemotherapy and radiation therapy [34].

St. Croix and colleagues have compared gene expression patterns of endothelial cells derived from blood vessels of normal and malignant colorectal tissues [35]. Isolation techniques of highly purified endothelial cells had to be developed, combined with a technique of mRNA transcript quantitation not dependent on pre-existing databases of expressed genes, since in these databases transcripts from endothelial cells are not well represented given the small fraction of endothelial cells in tissues from which the databases are derived. Three novel and abundantly expressed pan-endothelial markers or PEMs and 14 novel tumour endothelial markers or TEMs were discovered. Clinical studies aimed at revealing their prognostic or predictive value and their usefulness as a therapeutic target are ongoing.

3. Methodology of angiogenesis quantification in solid tumour sections

Counting microvessels in a microscopic field of a tumour tissue section gives an estimate of the net result of phases of angiogenesis and of the angioregression a tumour went through. The rationale of counting microvessels in vascular 'hot spots', or in areas giving the impression at low magnification of containing numerous microvessels, is that these areas originated from tumour cell clones with the highest angiogenic potential and, consequently, with the easiest access to the blood stream and with an increased probability of producing metastasis capable of becoming angiogenic and growing tumours. Hypoxia is indeed a strong angiogenic drive and a selection pressure resulting, for instance, in the survival of *TP53*-mutant tumour cells with a deficient apoptotic pathway prone to other oncogenic mutations [39]. In *in vitro* conditions, *TP53*-mutant tumour cells have been shown to produce more VEGF than cells with an intact *TP53* gene [40]. Although correlation analysis in human tumours between mutant *TP53* and angiogenesis has not confirmed the *in vitro* observation of p53-regulation of VEGF, this may be because only crude and not spatial analyses have been performed. Tumour cells with a deficient hypoxia-responsive pathway have been shown to have a survival advantage by virtue of the ability to select clones that are less dependent on the presence of blood vessels or on angiogenesis [41]. Therefore, a measure of the hypoxic responsiveness in human tumours such as using a combination of microvessel density and hypoxia-inducible factor (HIF) immunostaining might identify those neoplasms that are less responsive to treatment based on anti-angiogenesis or vascular targeting.

The Chalkley point overlap morphometric technique [42] has abolished one of the highly observer-dependent steps of measuring microvessel density, namely the frequent decision an observer had to make whether two immunostained and adjacent structures were the reflection of one single or two separate blood vessels. The Chalkley count is the number of grid points that hit stained microvessels. It is a relative area estimate rather than a true vessel count. The use of this quantification technique has led to large studies of breast cancer patients showing a very significant and independent prognostic value of Chalkley microvessel count in vascular hot spots for survival. Hansen and colleagues have studied the prognostic value of angiogenesis quantification by Chalkley counting in 836 patients with invasive breast carcinomas with a median follow-up time of more than 11 years [43]. The mean value of the counts in the three most vascular areas was taken for further analysis. Independent of other prognosticators, a 57% higher risk of dying was observed when the tumour had a Chalkley count between $\geqslant 5$ and <7. A 125% higher risk of dying was associated with a Chalkley count $\geqslant 7$. The Chalkley count predicted overall and disease-free survival, both in the node-negative and in the node-positive patient groups.

In carcinomas, and not necessarily in sarcomas [44], microvessels cluster in so-called 'vascular hot spots'. The most observer-dependent step is the selection of these densely vascularised areas in a tumour tissue section. This has led to the negative judgement of the College of American Pathologists concerning microvessel density as a prognostic factor in breast cancer [15]. Only in a few studies have attempts been made to solve this problem.

Beliën and colleagues have used automated image analysis algorithms in combination with a motorised scanning stage microscope and an autofocus device to scan whole tumour sections [45]. After immunohistochemical staining of tumour sections with antibodies directed at CD31 encompassing an additional incubation with biotinylated tyramine, a software program was developed to identify microvessels in whole tumour sections, generating a geographical microvessel map. The program was made to search these maps for vascular hot spots of user-defined shape and size, allowing for fully automated and thus more objective microvessel counting, overcoming most of the problems of subjectivity discussed in the previous consensus report. The developers of the method mentioned a throughput of the system of an average of 3.5 h per square centimetre, reducible by optimisation of the system to 1.4 h per square centimetre. The time-consuming nature together with the specialised equipment needed to perform the

analyses are the major drawbacks of this method, hampering its widespread application. Obviously, a comparison with the Chalkley counting method in a large prospective study in breast cancer has to be performed.

Endothelial cell proliferation (ECP) is measured after double immunostaining of tumour tissue sections with antibodies directed at an endothelial cell marker and a marker of proliferating cells. This technique allows for the simultaneous assessment of endothelial and tumour cell proliferation (TCP). The ratio of tumour to endothelial cell proliferation (TCP/ECP) roughly reflects the degree of angiogenesis-independent tumour growth. Liver metastases with a replacement growth pattern, co-opting the liver stroma with the sinusoidal blood vessels, have a high TCP/ECP ratio [6] while primary colorectal and breast adenocarcinomas have a TCP/ECP ratio of approximately 8, independent of the tumour histiotype [25]. ECP might be of interest to select tumours for the application of treatment schedules designed to inhibit blood vessel growth.

Another parameter reflecting the functional status of the microvascular bed is the amount of pericyte recruitment. Eberhard and colleagues have measured the pericyte coverage index of microvessels in six different types of malignant human tumours [46], by simultaneous staining of endothelial cells (anti-CD34 or anti-vWF) and mural cells (anti-alpha-smooth muscle actin). At least five independent microscopic fields per section were independently analysed by two investigators. The fraction of microvessels covered by pericytes ranged from 10% (median value) in glioblastomas to approximately 70% in colon carcinomas. In follicular development within the corpus rubrum of the ovary, a phase with high physiological angiogenic activity, 60% of the blood vessels expressed alpha-smooth muscle actin. An apparent lack of correlation between a high endothelial cell proliferation fraction and a low pericyte coverage index was observed, probably based on the pre-existing differences of pericyte coverage index of the surrounding normal tissue in the different tumour histiotypes. Data on pericyte coverage of tumour microvessels will have to be interpreted keeping in mind the coverage index of the respective normal parenchyma. Blood vessel remodelling, as has been shown in the postnatal vasculature of the retina [47], is only possible in the absence of a pericyte coating. This observation has important implications for anti-angiogenic treatment. Benjamin and colleagues [48] have indeed shown that androgen ablation therapy of prostate tumours, leading to downregulation of VEGF within the tumour, induces selective regression of microvessels that were not covered by pericytes. Blood vessel maturation parameters might predict the efficacy of an anti-VEGF treatment in reducing the tumour mass in individual patients.

Treatment monitoring of anti-angiogenic drugs in humans will have to rely on the quantification of regression of blood vessels. In addition to comparing pretreatment and posttreatment microvessel density, the fraction of apoptotic endothelial cells directly reflects the effect of treatment on endothelial cell survival. Treatment of animals bearing xenografted human tumours with selective inhibitors of the VEGF receptor, resulted in significantly increased levels of endothelial cell apoptosis [49]. Apoptotic endothelial cells were detected by combining immunohistochemistry for CD31 with the TdT-mediated dUTP-biotin nick-end labelling (TUNEL) method. The numbers of apoptotic endothelial cells were scored in five randomly chosen fields/section. Detailed staining protocols are provided in the respective manuscript. An alternative method would be to double-immunostain tumour sections with antibodies directed at endothelial cells and at single-stranded DNA [50].

Table 2 summarises the current consensus methodology to assess the different angiogenic parameters in solid tumours. Staining protocols are given in Table 3.

4. Surrogate histopathological markers of the angiogenic activity of tumours

Histological surrogate markers should give a reliable, fast and technically easy estimate of the amount of angiogenesis, ongoing and past, of a tumour. Ideally, a standard histochemical technique, such as a haematoxylin-eosin stain, should suffice. Interpretation of the slides should not rely on extensive training and should not be time-consuming. Yet, a clear pathophysiological mechanism should corroborate the association of these markers with parameters directly reflecting angiogenesis, such as microvessel density, the fraction of proliferating endothelial cells and the degree of pericyte-coverage of microvessels in the tumour tissue. Two candidate surrogate markers of angiogenesis are: the fibrotic focus and the growth pattern.

A fibrotic focus is defined as a scar-like area replacing necrosis in the centre of a carcinoma. It was proposed in 1996 by Hasebe and colleagues [51] as an indicator of tumour aggressiveness in invasive ductal carcinoma of the breast. It appears as a radially expanding fibrosclerotic core and consists of loose, dense or hyalinised collagen bundles and a variable number of fibroblasts. Elastic tissue may be abundant. Fibrotic foci smaller than 3 mm in diameter do not contain carcinoma cells, while larger fibrotic foci sometimes do. Remnants of necrotic tissue may still be present inside the fibrotic focus. In breast cancer, the presence of a fibrotic focus was shown to predict higher microvessel density and a higher fraction of proliferating endothelial cells [52,53]. Although confirmatory studies are required, it appeared that the presence of a fibrotic focus was significantly associated with early distant relapse in node-negative breast cancer patients [54].

Table 2
Standard methods for angiogenesis quantification in solid tumour sections

1. Relative microvessel area estimate ('Chalkley count')
Unit: no
Methodology:
• Immunostaining with antibodies against CD34 of one representative tumour section.
• Chalkley point-overlap morphometry: mean of three most vascular areas within the tumour (×200 magnification) after manual selection of these areas ('hot spots') by scanning the whole section at low magnification.
Remarks:
• Training necessary for the hot spot selection.
• No clear hot spots in sarcomas.
Improvements:
• Quality control should be performed under the auspices of international collaborative groups: distribution of reference slide sets.
• Widely applicable and fast automated hot spot selection: to be compared with manual hot spot selection in large prognostic/predictive studies.
• To be compared with presence or absence, size, and composition of fibrotic focus in large prognostic/predictive studies.
• Estimation of the size of the viable areas within a tumour with an extremely low Chalkley count as an indicator of less angiogenesis-dependent growth?

2. Endothelial cell proliferation fraction
Unit: percentage
Methodology:
• Double-immunostaining with antibodies against CD34 or CD31 and against Ki67.
• Random fields.
Remarks:
• To be tested as a parameter to predict individual response to anti-angiogenic drugs or as a parameter to monitor response to these drugs: to be compared with growth patterns in this context.
Improvements:
• To be evaluated together with the endothelial cell apoptosis fraction after establishment of a reproducible double-staining technique using anti-single-stranded DNA antibodies.
• Is the evaluation of endothelial cell proliferation and of endothelial cell apoptosis fraction on core biopsies representative for the whole tumour?

3. Pericyte coverage index
Unit: percentage
Methodology:
• Double-immunostaining with antibodies against CD34 or CD31 and against alpha-smooth muscle actin.
• Random fields.
Remarks:
• To be tested as a parameter to predict individual response to anti-angiogenic drugs or as a parameter to monitor response to these drugs: to be compared with growth patterns in this context.
• To be evaluated in relation to the pericyte coverage index of adjacent normal tissue.

A second histological surrogate marker for angiogenesis is the growth pattern of tumours. Both in liver metastases and in primary and metastatic lung tumours, growth patterns with distinct angiogenic profiles were described [3,4,6]. In primary breast cancer [54] and in cutaneous breast cancer deposits, different growth patterns also reflected differences in angiogenesis (data not shown). In the infiltrative growth pattern, the carcinoma cells infiltrated between pre-existing dermal structures, including collagen bundles, blood vessels and skin adnexa, without significant disturbance of the dermal architecture. In the expansive growth pattern, the dermal deposit formed a well circumscribed nodule consisting of carcinoma cells and desmoplastic connective tissue. Pre-existing dermal structures were not present inside the deposit, but were pushed aside by the expansively growing nodule. In the expansive growth pattern, the endothelial cell proliferation fraction, the Chalkley count and the amount of fibrin were significantly higher compared with the infiltrative growth pattern. For the evaluation of both the fibrotic focus and the growth pattern, only a routine haematoxylin-eosin stain is needed.

A promising immunohistochemical marker of the degree of angiogenesis-dependent growth of tumours might be the expression of hypoxia-inducible factor or HIF-1. Antibodies directed at the HIF-1α subunit, suitable for staining of paraffin sections, have been developed [55,56]. In epithelial ovarian tumours, HIF-1α expression was observed in approximately 70% [57]. Expression of the protein was determined semi-quantitatively by assessing the percentage of decorated tumour cell nuclei and the staining intensity. Although in particular tumour cells directly adjacent to necrosis strongly expressed HIF-1α, well-differentiated ovarian cancers showed an overall stronger expression of HIF-1α protein than poorly differentiated tumours. In addition, a higher percentage of non-invasive borderline tumours showed HIF-1α expression when compared with invasive cancer. This probably suggests that HIF-

Table 3
Immunohistochemical staining protocols

1. Anti-CD34 for Chalkley count
- Paraffin-embedded formalin-fixed tissue, 5 μm sections, no antigen retrieval pretreatment, endogenous peroxidase blocking.
- Anti-CD34 monoclonal antibody (Biogenex, clone QBEND/10), dilution 1/5 in 1% phosphate-buffered solution/bovine serum albumin (PBS/BSA), overnight incubation at room temperature.
- Goat-anti-mouse (Dako E0433), dilution 1/400, 30 min at room temperature.
- Strept ABC (Dako K0377), according to protocol supplied.
- Chromogen DAB (Liquid DAB Concentrated Substrate Pack, Biogenex HK-153–5K), according to protocol supplied.
- Counterstaining: haematoxylin.

2. Anti-Ki67-anti-CD31 for counting proliferating endothelial cells
- Paraffin-embedded formalin-fixed tissue, 5 μm sections, microwave heating (30 min at 90–95 °C in ethylene diamine tetraacetic acid (EDTA) pH 9, Antigen Retrieval Solution, Dako PP 20–0226).
- Endogenous peroxidase blocking.
- Anti-Ki67 polyclonal antibody (Dako N1574), prediluted, 30 min at room temperature.
- Goat-anti-rabbit (Dako E0432), dilution 1/500, 30 min at room temperature.
- Strept ABC and DAB as described before.
- Anti-CD31 monoclonal antibody (Dako M0823, clone JC/70A), dilution 1/20, 32 min on a Ventana NexES automated immunostainer at 37 °C (760–030, Ventana Medical Systems, Inc.).
- Ventana Basic Alkaline Phosphatase Red detection kit.
- Counterstaining: haematoxylin.

3. Anti-CD34-anti-alpha-smooth-muscle-actin for estimation of the fraction of immature blood vessels
- Paraffin-embedded formalin-fixed tissue, 5 μm sections, no antigen retrieval pretreatment, endogenous peroxidase blocking.
- Anti-CD34 immunostaining as described before.
- Anti-alpha-smooth muscle actin monoclonal antibody (Biogenex, MU128.UC, clone 1A4), dilution 1/80, 32 min on a Ventana NexES automated immunostainer at 37 °C (Ventana Medical Systems, Inc.).
- Ventana Basic Alkaline Phosphatase Red detection kit.
- Counterstaining: haematoxylin.

4. Anti-PAL-E- anti-CD31 to differentiate blood and lymphatic vasculature
- Frozen 4 μm sections fixed in acetone for 10 min.
- Undiluted PAL-E mAb supernatant (Sanbio, Uden, The Netherlands), 1 h.
- Biotinylated horse anti-mouse Ab (Vectastain), dilution 1/200, 30 min.
- ABC-peroxidase (Vectastain), dilution 1/100, 45 min.
- AEC, 10 min.
- Anti-CD31 mAb (British Biotechnology, ITK Diagnostics, Uithoorn, The Netherlands), dilution 1/2000, 1 h.
- Rabbit anti-mouse conjugated with alkaline phosphatase (Dako), dilution 1/40, 30 min.
- Fast Blue (Sigma), 20 min.
- Counterstaining with haematoxylin.

All procedures are at room temperature.

1α expression is not a simple marker of hypoxia, given the higher degree of hypoxia expected in poorly differentiated ovarian cancers versus well-differentiated invasive tumours and versus non-invasive borderline tumours. The vast majority of specimens with a cystadenoma were HIF-1α-negative. Microvessel density showed a correlation with HIF-1α expression in invasive and borderline cancers ($r = 0.62$, $P < 0.001$, $n = 102$ and $r = 0.77$, $P < 0.001$, $n = 50$). In tumours with strong expression of HIF-1α and p53 overexpression, microvessel density was significantly increased and the apoptotic rate was very low. Evaluation of HIF-1α expression by immunohistochemistry in brain tumours showed that protein expression was strongest in glioblastomas and in haemangioblastomas [55]. These are, respectively, the most malignant and the most vascularised primary tumours arising in the central nervous system. Comparison of tumour and normal flanking tissue allows the patient to serve as his own control since in normal tissue HIF-1α expression is absent. Talks and colleagues have raised another monoclonal antibody to investigate the distribution of HIF-1α by immunohistochemistry [56]. While sections from breast adenocarcinoma showed predominantly nuclear immunostaining, in sections of hepatocellular carcinoma, nuclear and cytoplasmic staining was demonstrated. The distribution of *VEGF* mRNA correlated with the protein distribution of HIF-1α.

Hypoxia in human cervix carcinomas, measured using an Eppendorf needle electrode, correlated, albeit weakly ($P < 0.005$; $r = 0.58$), with intercapillary distance (ICD), but not with microvessel density [58]. ICD and microvessel density provided independent prognostic information in a multivariate analysis. To measure ICD, a cross-hair in the eye-piece of a microscope was superimposed over the field of view and the centre positioned over a vessel. Using an image analysis system, the distance between the central vessel and the nearest vessel in

each of the four quadrants was measured. This was repeated in adjacent fields of the whole tumour section and mean values were used for the final analyses.

5. Other surrogate markers of tumour-related angiogenesis

The main disadvantage of the histological surrogate markers of angiogenesis is the inherent inter-observer variability. A more objective approach is the quantification by multi-parameter flow cytometry of circulating endothelial cells [59]. In a mouse model of human lymphoma, the fraction of CD45- and Flk+ cells in the peripheral blood was found to represent circulating endothelial cells which also expressed CD31, VE-cadherin and MECA-32. In control mice, approximately 1 circulating endothelial cell was present per μl of blood. Most of these cells were apoptotic. In tumour-bearing mice, a mean of 10 circulating endothelial cells were found per μl of blood. Most of these cells were viable. The number of circulating endothelial cells correlated with the tumour volume and with the concentration of VEGF in the blood. The correlation with microvessel density was slightly weaker. After endostatin-treatment of the lymphoma-bearing mice, most of the circulating endothelial cells were apoptotic. Cytotoxic drug treatment, on the other hand, did not increase the fraction of apoptotic endothelial cells in the peripheral blood. The measurement of the viability of circulating endothelial cells by flow cytometry might thus be relevant in evaluating the anti-angiogenic activity of different drugs.

Circulating levels of VEGF have been measured by several investigators who reported mixed results [60–86]. Discrepancies may be related, at least in part, to a lack of standardisation of the pre-analytical phase.

Circulating VEGF has a multi-compartmental origin; it may be related to the tumour, but it is certainly produced and released also by platelets, granulocytes, monocytes, mast cells and lymphocytes [87–94]. A direct relationship between platelet count and serum VEGF concentration has been found by several investigators [79]. Verheul and colleagues [87] showed that VEGF is transported by platelets and serum VEGF concentrations reflect platelet counts rather than tumour burden. Maloney and colleagues [89] and Gunsilius and colleagues [93] reported a clear association between platelets aggregation *in vitro* and VEGF levels and showed that VEGF released from platelets during blood clotting is the main source of VEGF in serum samples.

Time from the venipuncture to centrifugation ranges from 'immediately' to 30 min at room temperature and from 30 min to 4 h at 4 °C. Not surprisingly, serum VEGF concentrations found in healthy subjects are widely scattered also when the same assay method was used, ranging from 0 [60,69,76,87] to 1750 pg/ml. Recently, Dittadi and colleagues [95] showed that serum VEGF increases in a variable manner over 2 h during clotting at room temperature, while it became quite stable thereafter; the increase ranged from 1.2 to 45.2-fold with reference to VEGF level found in the serum obtained 10 min after blood withdrawal.

Clinical studies in which plasma was used present also a wide variety of modality for sample collection and manipulation. Few studies dealt with standardisation of sample collection and processing. Banks and colleagues [88] described an optimised procedure to process samples for the determination of circulating VEGF. They evaluated several anticoagulants and blood collection tubes. They concluded that the optimal procedure was sodium citrate plasma processed within 1 h from venipuncture. However, they handled samples at room temperature and did not monitor markers of *in vitro* platelet activation. Of note, measurable levels of VEGF were found in 7/8 of their healthy volunteers, suggesting an *in vitro* activation of platelets with release of VEGF. Recently, Adams and colleagues [82] used the procedure described by Banks and colleagues to study VEGF levels in 201 subjects, including 53 cases with primary breast cancer. They found that plasma VEGF was more effective than serum VEGF in discriminating cancer from both controls and patients with benign diseases. The median VEGF level found in healthy controls was 27.3 pg/ml. Using the same technique of plasma preparation, Dittadi and colleagues [95] found high plasma levels of platelet factor 4 (PF4); therefore, the values that they found in controls might be due to a partial *in vitro* activation of platelets, making their conclusion on the role of plasma VEGF as a tumour marker questionable.

Wynendaele and colleagues [92] performed a study using different blood derivatives, including CTAD (buffered tri-sodium Citrate solution with Theophylline, Adenosine and Dipyridamole) plasma. They recommend that CTAD plasma should be used for the VEGF assay; however, they also reported that the best discrimination between 18 cancer patients and 22 controls was platelet-poor plasma. Notably, they found median PF4 values of 102 and 103 IU/ml in platelet-poor plasma of volunteers and patients, respectively. These PF4 values indicate an *in vitro* activation of the platelets before preparation of platelet-poor plasma since, as the authors themselves declared, PF4 plasma levels in normal adults should be 0–5 IU/ml. Accordingly, when using CTAD plasma they found lower PF4 levels. They did not comment on the fact that they had prepared platelet-poor plasma handling citrated blood at room temperature. Wynendaele and colleagues possibly emphasised the putative diagnostic information provided by VEGF measured in an artefactual blood derivative (platelet-poor plasma in which a partial platelet activation occurred).

Dittadi and colleagues [95] compared sodium citrate plasma, prepared according to routine procedures, with plasma obtained using two procedures (Edinburgh and the CTAD mixtures) aimed at avoiding in vitro platelet activation. They used the platelet specific PF4 to monitor the rate of *in vitro* platelet activation. Both of the procedures were effective in preventing release of PF4 and VEGF by platelets. By contrast, plasma citrate could not prevent release of both PF4 and VEGF from platelets even in stringent sampling conditions; therefore, it should not be used for VEGF determination.

VEGF in the different blood compartment could have a different biological and clinical meaning. In agreement with data reported in several papers [79,88–91,93], serum VEGF was correlated with platelet count, but not with leucocytes; therefore, serum VEGF, if obtained with a standardised procedure, should provide information on the capability of the platelet pool to release the marker. Evidence available so far does not indicate the most appropriate blood compartment in which to measure VEGF. Therefore, we suggest that VEGF should be systematically evaluated in CTAD plasma, in serum and in whole blood. Prospective controlled clinical studies are necessary to clarify in which blood compartment(s) VEGF will eventually provide clinically relevant information. A standardised procedure for sample collection should be used in clinical studies aimed at evaluating circulating VEGF in order to avoid pre-analytical biases that may affect the interpretation of clinical results. In addition, the assay of PF4 should be routinely performed to monitor *in vitro* platelet activation when evaluating plasma VEGF.

A proposed standard methodology to measure circulating VEGF is given in Table 4.

VEGF concentration in tumour tissue was significantly higher in glioblastomas compared with other tumours, as well as in normal brain. There was no correlation in VEGF concentration between the serum and tissue extracts. Microvessel density correlated with VEGF concentration in the tumour tissue ($r = 0.76$; $P = 0.003$; $n = 17$) [86]. In endometrial carcinoma, higher cytosol VEGF concentrations and a higher microvessel density were noted in tumours with advanced stage ($n = 53$) [96]. The cytosol VEGF and microvessel density showed a positive linear correlation ($r = 0.41$; $P = 0.003$). In a study of 135 breast carcinomas, the average VEGF concentration in tumours with more than 100 microvessels/mm^2 was 227 pg/mg protein [97], while in less vascular tumours it was 86 pg/mg protein ($P < 0{,}01$).

Subsequently, the method was modified by Gasparini and colleagues [98] to detect VEGF protein in frozen cytosol using the same samples routinely adopted for determination of steroid hormone receptors. The prognostic value of cytosolic concentrations of VEGF was studied both in a series of node-negative and node-positive patients [98–100]. In all of the studies published

Table 4
Circulating VEGF assay

Proposed standard protocol for the collection of blood samples and blood derivative preparation.

1. Sampling
- Apply stasis to the vein for the shortest possible time.
- Release the tourniquet when blood begins to flow.
- Collect 12 ml of blood in a prechilled syringe.

2. Serum
- Draw 5 ml of blood in plain polystyrene tube.
- Allow the sample to clot for 30 min at 37 °C.
- Let the sample stand for 30 min at room temperature and centrifuge at 1500 *g* for 10 min.

3. Plasma
- Break the vacuum of the CTAD tube.[a]
- Draw 4.5 ml of blood in the CTAD tube.[a]
- Mix the tube by gentle inversion.
- Place the tube immediately into the ice bath.
- Centrifuge at 1500*g* for 10 min at 4 °C within 1 h.
- Carefully pipette one-third of the supernatant plasma from the centre portion of the liquid phase.
- Do not remove liquid from the top layer (which contains low-weight platelet components) or from the region adjacent to the cellular layer.

4. Whole blood
- Dispense 2.5 ml of blood in a test-tube containing 0.1 ml of sodium citrate (final concentration 3.1 mg/ml).
- Add 5 ml of sterile water, mix and freeze-thawing the samples twice (store at −80 °C overnight/thaw at room temperature).
- Mix thoroughly before stocking the aliquots.

5. Storage
- Stock serum, plasma and whole blood in 1.5 ml polythene tubes.

snap freeze in liquid nitrogen (or in ether/dry ice).
- Store at −80 °C.

[a] CTAD tubes: (sodium Citrate, Theophylline, Adenosine, Dipyridamole).

by Gasparini and colleagues [98–100], as well as from most of the other authors [101–108], high VEGF levels in tumour tissue were found to be significantly associated with poor prognosis and poor benefit of conventional adjuvant treatments (reviewed in [109,110]).

Levels of circulating angiogenic factors might in the future be applied in clinical practice to monitor response to cancer therapy, although confirmatory studies are required (reviewed in [111]). Dirix and colleagues [83] were the first to show that serum VEGF and basic fibroblast growth factor (bFGF) levels were higher in progressive disease compared with responsive disease in patients with metastatic cancer from various origins treated with chemotherapy. A small number of studies with patients treated with different combinations of chemotherapy, radiotherapy, and even gene therapy has confirmed these results (reviewed in [112]).

6. Quantification of lymphangiogenesis

In several types of malignant tumours, like cutaneous melanoma and head-and-neck squamous cell carcinoma, the microvascular density appears not to correlate with metastatic spread [113–115]. Until now, the reason for this lack of correlation is unclear, but it may be speculated that, next to several properties of tumour cells and tumour-associated stromal cells, the presence of a lymphatic vascular bed is an important factor. It is obvious that the propensity of a tumour to spread initially via the lymphatic vascular bed may be dependent on a high intrinsic lymphatic density in the tissue where the tumour arose, and, indeed, in the skin and in the head and neck area, this is the case. However, in breast tissue lymphatic density is high as well, but still this is the classical example of a tumour type where blood capillary density is strongly related with prognosis, i.e. metastasis [116]. It should be realised, however, that in spite of the correlation with blood vessel density, this specific tumour type has an initial propensity to spread towards draining lymph nodes as well.

In view of these considerations, it seems obvious that there are complex and partly unknown relationships between tumour lesions, blood and lymph vessels. On the role of lymphatic vessels in this interplay, there is only patchy knowledge, because of a lack of widely available specific markers for lymphatic endothelium, and of easily available and reliable cultures of lymphatic endothelial cells. Furthermore, the lymphatic bed is generally regarded as a passive system, draining excess fluids and transporting cells, not exerting vital and dynamic functions like the blood vessel system. Therefore, a detailed investigation has probably not been considered to be worthwhile. Recently, however, potential new markers for lymphatic endothelium have been described, and reports on the possible occurrence of lymphatic angiogenesis were published [117–121].

The overall conclusion from these recent studies was that haemangiogenesis (blood vessel formation) in tumour lesions is driven by VEGF-A, and is associated with haematogenic metastasis, and that, conversely, lymphatic spread is preceded by lymphangiogenesis, which is a process driven by VEGF family members VEGF-C and D. The strong relationship between blood vessel density and prognosis in mammary carcinoma, although this tumour often spreads via the lymphatics, indicates that the situation is probably more complex.

A possible candidate marker for lymphatic immunostaining is the VEGF-receptor-3, also known as Flt-4 [117]. In adult life, Flt-4 is restricted exclusively to the lymphatic endothelium [117–121]. However, recent studies demonstrated that this receptor is upregulated on blood vessels in carcinoma [122], making this marker less useful for the detection of lymphatics in tumours, although it has been used for this purpose [123,124]. In invasive breast cancer, a loss of the long, but not of the short, isoform of Flt-4 was observed compared with normal breast tissue [125]. This difference was largely accounted for by the reduction of long-Flt-4 in lymph node-positive tumours. Lymphangiogenesis might thus be controlled by alternative splicing of Flt-4 in breast cancer. Another marker that was expressed specifically on lymphatic endothelium is podoplanin [126,127]. This molecule is a membrane glycoprotein that was identified on podocytes in rat kidneys [128] and was expressed in endothelial cells of normal lymphatics and benign lymphatic tumours [126]. However, the antibody is not widely available. A next possible marker is desmoplakin: this junctional marker can be used in frozen sections [129], but is expressed on various other cell types as well. In addition, it has been reported that lymphatics can also be evaluated by 5′-nucleotidase enzyme histochemistry [130–132]. Finally, very recently, the LYVE-1 marker [133] was used successfully for lymphatic detection [134,135], but this marker is until now not commercially available as well. LYVE-1 is a lymphatic-restricted receptor for hyaluronan, a matrix glycosaminoglycan.

Extensive analyses of series of tumours for localisation of lymphatics and lymphatic density are scarce thus far. One recent report describes lymphatic microvessel density as a novel prognostic factor in early-stage invasive cervical cancer, using an antibody against podoplanin [136].

We have described a double staining protocol that differentially stains blood and lymphatic vasculature using the blood vessel endothelial marker PAL-E and pan-endothelial marker CD31 in frozen sections of normal skin and cutaneous melanoma [137]. The staining protocol is given in Table 3. Subsequent studies

Table 5
Potential clinical use of angiogenesis quantification[a]

Parameter and method	Potential clinical use	Implementation	Main advantage
• Relative area estimate of blood vessels (CD34-immunostaining and Chalkley point overlap morphometry)	• OS and DFS-prediction in node-negative/node-positive breast cancer	2	Less observer-dependent than microvessel counting
• Endothelial cell proliferation fraction (double immunostaining: CD31-Ki67)	• Prediction of efficacy of anti-angiogenic treament directed at activated/proliferating endothelial cells	1	Reflection of ongoing angiogenesis
• Pericyte coverage index (double immunostaining: CD34-alpha-smooth-muscle-actin)	• Prediction of efficacy of anti-VEGF or VEGF withdrawal therapy	1	Reflection of the functional status of the vasculature with respect to remodelling
• Endothelial cell apoptosis fraction (methods still to be developed and compared)	• Treatment monitoring of anti-angiogenic drugs on repeated biopsies in a neo-adjuvant setting	1	Direct measurement of endothelial cell death: early marker?
• Presence of fibrotic focus (standard haematoxylin-eosin (HE) stained slide)	• Surrogate marker of high Chalkley count: prognostic factor in breast cancer	2	Easier than Chalkley or counting blood vessels
• Growth pattern (HE-stained slide)	Detection of angiogenesis-independent growth: resistance to anti-angiogenic treatment?	1	Simple to perform
• Concentration of circulating VEGF (ELISA or comparable techniques: blood, serum or plasma)	• Treatment monitoring of chemo-, radio- and gene-therapy • Target detection and treatment monitoring of anti-angiogenic drugs	2	• Successive measurements • Integrated information of all tumour foci • Observer-independent method
• Viability and number of circulating endothelial cells (flow cytometry)	• Surrogate marker of angiogenesis (number) • Monitoring of response to anti-angiogenic treatment (viability)	1	• Observer-independent method

OS, overall survival; DFS, disease-free survival; ELISA, enzyme linked immunosorbent assay.
[a] Phase of implementation: 1, still experimental; 2, convincing data that suggest that the parameter and method might be implemented in the near future-confirmatory (multicentre) study needed.

demonstrated that this double staining protocol could be extended to a variety of other types of normal and tumour tissues [138], making this staining protocol a reliable tool to evaluate the presence of lymphatic vessels. Using this approach, both blood and lymph vessel density were determined in a series of cutaneous melanoma lesions by counting the number of stained structures per microscopic field at random. There was no selection of vascular hot spots because often the primary lesions were limited in size. Both intra- and peritumour vessels were included in the countings. Blood vessel density was increased in the vertical growth phase, but lymphatic density remained at control levels. However, in rare, individual cases, a strong increase in lymphatic density could be observed (data not shown).

Based on these results, the role of the lymphatic vessels in cancer can be summarised as follows: (1) Tumours that arise in tissues with a high intrinsic lymphatic density have a high chance of lymphatic spread. The rate of lymphatic metastasis will be determined by the level of expansion of the primary lesion. Haemangiogenesis, induced by VEGF-A expression, but maybe also by the expression of other VEGF family members, may be an important driving force for this process. Cutaneous melanoma is an example of this type of situation, which potentially leads to both haematogenous and lymphatic spread. (2) Tumours may induce lymphangiogenesis via expression of VEGF-C and -D. This may occur in tissues with both high and low lymphatic density. This process may favour lymphatic spread specifically. Thus far, proven examples of this situation in human tumour tissues are lacking, but the recent experimental evidence shows that it may occur. (3) Tumours may arise in tissues lacking lymphatic vessels, like the eye or the brain. Recent data show that lymphangiogenesis in this situation is very unlikely to occur, even in the presence of a lymph vessel growth factor like VEGF-C [138].

7. Summary and future directions

With the development of angiogenesis inhibitors—over 150 compounds are in different stages of clinical development from phase I up to phase III studies—detailed knowledge of the vascular component of a

tumour in a particular patient is becoming essential. Most drugs target one component of the angiogenesis program, yet the studies reviewed above show the great inter- and intra-individual variability in the process. It is perhaps not surprising that, as of yet, marked tumour regressions in many patients have not been reported. The following information is likely to affect the activity of the antivascular treatment: the degree of angiogenesis-(in)dependent growth, the intensity of ongoing angiogenesis, and the dominant pathway involved. A fair evaluation of an anti-angiogenic compound in a clinical setting also implies detailed knowledge of the mechanism of action of a particular drug. This seems to be the case for a larger part of the anti-angiogenics, such as anti-VEGF receptor tyrosine kinase inhibitors or anti-VEGF antibodies, but for the much publicised so called natural angiogenesis inhibitors endostatin, angiostatin and vasculostatin, this is much less obvious. If a drug acts on a particular target, this target should be measured in the primary tumour and, if possible, in the metastases. The response can then be correlated with the mechanism. The presence of particular targets in secondary deposits needs further study. However, recent studies with specific positron emission tomography (PET) scanning have validated the predictive value of oestrogen receptor or c-erbB-2 overexpression in metastasis, as revealed by their presence in the primary tumour [139,140]. Indirect evidence for a VEGF-mediated pathway are (1) the presence of contrast enhancement and delayed wash-out curves on standardised computed tomography (CT)-scanning, reflecting increased vascularity and/or permeability, and (2) the presence of pleural and peritoneal effusions. In this approach, treatment of patients can be based on a particular presentation of their cancer rather than on the histiotype of their disease. Tumour size, tumour site and the presence or absence of the primary tumour in the case of metastases might influence the mediators and the intensity of angiogenesis. Resection of metastases, a procedure gaining widespread acceptance for lung, liver and brain metastases, provides the opportunity to investigate this.

Repeated tumour biopsies in preclinical models have demonstrated that active anti-angiogenesis leads to a decrease in microvessel density, and to an increase in endothelial cell apoptosis and secondary tumour cell apoptosis. Although in clinical conditions repeated biopsies to estimate the effect of treatment can be difficult to perform and might be hampered by tumour heterogeneity, in a neo-adjuvant strategy, used more and more in common solid tumours such as breast cancer, non-small cell lung cancer, rectal cancer and oesophageal cancer, this should be a feasible option. Comparing pre- with posttreatment biopsies will be the only way to define a set of angiogenesis parameters that will predict response to particular anti-angiogenic drugs. There is for instance no convincing evidence that high microvessel density predicts susceptibility for an anti-angiogenic approach. In addition, the so-called angiogenesis-independent growth patterns have not been tested for their (lack of) response. Studies to determine whether microvessel density measured in core biopsies of primary breast cancer is representative of overall vascularity have been performed and have failed to demonstrate a positive correlation in microvessel counts in core biopsies and whole tumour sections. In a study from Edinburgh, microvessel counts were compared in core biopsies and tumour sections taken simultaneously at the time of surgery ($n=16$) and, whilst the range of counts was similar in the two groups, correlation between counts failed to reach statistical significance. Vessel counts tended to be higher in the tumour sections than in the core biopsies. In addition, microvessel counts in core biopsies were compared with counts in tumour sections taken at separate time-points between 14 and 21 days apart ($n=21$) and a similar lack of correlation was observed. An increase in the number of core biopsies taken did not improve the correlation [141]. Similar findings were obtained in an earlier study, that compared several variables in core biopsy versus tumour sections. Whilst variables such as oestrogen receptor, c-erbB-2, p53 and DNA ploidy correlated well in core biopsies and tumour sections, reproducible results were not obtained for microvessel counts and accuracy was not improved by increasing the tumour area available by performing more core biopsies [142]. Studies comparing the growth pattern of breast cancer, infiltrative or expansive, in core biopsies and in the respective whole tumour sections are ongoing.

In conclusion, reliable angiogenesis parameters are urgently needed in the emerging therapeutic setting of anti-angiogenesis. This second consensus report is intended to offer clinical investigators tools to develop or adopt techniques to measure several aspects of angiogenesis (Table 5). In the near future, however, high-density, membrane-based hybridisation arrays or 'micro-arrays' to study mRNA expression patterns might be helpful in identifying groups of genes predictive of response to anti-angiogenic treatment [143]. Clinical efficacy of 'angiostatic' or 'angioregressive' compounds will have to be clearly defined before such analyses can be performed successfully.

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