

## Evaluation of Vascular Proliferation in Molecular Subtypes of Breast Cancer

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**Abstract.** *Background: Angiogenesis plays a pivotal role in tumor development. Although microvessel density (MVD) is the most common method used for evaluation of angiogenesis, it has several limitations. Our aim was to evaluate MVD and microvessel proliferation (MVP) in a series of invasive breast carcinomas and analyze whether angiogenesis is influenced by the molecular phenotype of each tumor. Materials and Methods: We examined vascular proliferation using double immunohistochemistry (CD34/Ki67) in a series of 54 invasive breast carcinomas and compared the results with standard MVD, molecular subtypes and other classical parameters. Results: Increased MVD and MVP values were recorded in basal-like subtype, but only the MVP value reached significance among this group of patients ( $p=0.0001$ ). For all cases combined, increased MVP was significantly correlated with negative estrogen receptor (ER) status ( $p=0.010$ ) and higher histological grade ( $p=0.002$ ). Conclusion: MVP more accurately reflects the state of angiogenesis in breast cancer, compared with standard MVD. Vascular proliferation was associated with aggressive tumor features, indicating its contribution to tumor progression. The strong association between vascular proliferation and basal-like tumors suggests that this marker can be used for stratification of patients who might benefit from therapies targeting angiogenesis.*

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Angiogenesis is considered a hallmark of cancer and a key requisite in their growth, invasion and progression (1). In 1971, Folkman suggested that tumors can be treated by inhibiting their vascularization (2). It is well known that tumors cannot exceed 2-3 mm without vascular support (2), thus, anti-angiogenic therapy is an attractive target for angiogenesis-dependent tumors such as breast cancer.

Microvessel density (MVD) is the most widely method used for evaluation of angiogenesis, based on counting the vessels in the most vascularized areas of the tumors, namely 'hot spots'. This method was developed by Weidner and co-workers in 1991, who demonstrated that MVD influences the prognostic of patients with breast cancer (3, 4). Since then, many other researchers have investigated the role of MVD in breast tumors, but the results are contradictory. However, MVD has some limitations, as it cannot predict the response to therapy or the treatment efficacy (5).

Recent studies showed that microvessel proliferation (MVP), defined as the average number of vessels exhibiting co-expression of an endothelial and a proliferation marker, is a better indicator of angiogenesis compared with MVD (5-7). In prostate and endometrial carcinomas, microvessel proliferation was found to be a more reliable prognostic marker compared with standard MVD (5-7).

With this background, the aim of the present study was to evaluate vascular proliferation (CD34/Ki67 co-expression) and standard MVD in a series of invasive breast carcinomas, in accordance with the molecular classification. The results were compared by classical clinicopathological parameters.

### Materials and Methods

The present study included 54 female patients, aged between 39-85 years (mean=57.3 years), who underwent radical modified mastectomy and lymph node dissection between 2009-2013.

Surgical specimens were fixed in buffer formalin and paraffin embedded and 5  $\mu$ m-thick step sections were performed for each

case. The cases were graded according to the WHO Classification of Tumours of the Breast (8) and the Nottingham Grading System (9). Based on conventional histopathological examination, all cases included in this study were diagnosed as ductal invasive carcinomas of no special type (NST) type.

All procedures were carried out according to the principles of the Declaration of Helsinki and were approved by International Review Board of Victor Babeş University of Medicine and Pharmacy, Timișoara, Romania.

**Immunohistochemical procedure.** For immunohistochemical staining, we selected one representative slide from each case. The technique included heat-induced epitope retrieval with Bond Epitope Retrieval Solution 2, ready-to-use (Leica Biosystems, Newcastle Ltd, Newcastle Upon Tyne, UK) for 20 min. The immunohistochemical technique continued with the blocking of endogenous peroxidases using 3% hydrogen peroxide for 5 min. Sections were then incubated for 20 min with primary antibodies to: ER (clone 6F11, ready-to-use), progesterone receptor (PR; clone PGR 323, ready-to-use), human epidermal growth factor receptor 2 (HER2); clone CB11, ready-to-use), Ki67 (clone MIB-1, ready-to-use) and cytokeratin 5 (CK5; clone XM26, ready-to-use) all from Novocastra (Leica Biosystems, Newcastle Ltd, Newcastle Upon Tyne, UK). Bond Polymer Refine Detection System (Leica Biosystems) was used for visualization. 3,3 Diamino-benzidine dihydrochloride was applied as chromogen and hematoxylin was used for counterstaining.

Using immunohistochemistry, the cases were reclassified into four molecular subtypes as follows: ER<sup>+</sup>, PR<sup>+</sup>, HER2<sup>-</sup>, CK5<sup>-</sup> and Ki67<14% as luminal A; ER<sup>+</sup> with/without PR<sup>+</sup>, HER2<sup>+</sup>, CK5<sup>-</sup> or ER<sup>+</sup> with/without PR<sup>+</sup>, HER2<sup>-</sup>, CK5<sup>-</sup> and Ki67>14% as luminal B; ER<sup>-</sup>, PR<sup>-</sup>, HER2<sup>+</sup>, CK5<sup>-</sup> as HER2-overexpressing; ER<sup>-</sup>, PR<sup>-</sup>, HER2<sup>-</sup> and CK5<sup>+</sup> as triple-negative/basal-like (10). ER and PR were scored accordingly to the Allred system (11), and HER2 accordingly to American Society of Clinical Oncology recommendations (12). For the Ki-67 proliferation index we used a 14% threshold as the limit to define high/low proliferative cases (10).

Immunohistochemical study included double staining with CD34/Ki67. Heat-induced epitope retrieval with pH 6.0 solution (Leica Biosystems) for 30 minutes was followed by endogenous peroxidase blocking (3% hydrogen peroxide, 5 min). The procedure continued with incubation with primary antibody Ki67 (clone MIB-1, ready-to-use, 30 minutes; Novocastra), and then with the second antibody to CD34 (clone Qbend10, ready-to-use, 30 min; Novocastra), visualized with Warp Red as chromogen, for 10 min (Biocare Medical, LLC, Concord, CA, USA). The procedure was performed with Bond Refine Detection System DAB/RED.

**Assessment of neovascularization.** The hot-spot method was applied for MVD assessment (13). Therefore, the most vascularized areas of the tumor were selected and both proliferative and non-proliferative blood vessels were counted at ×400 magnification. Proliferating microvessels were counted in the same fields as MVD, at ×400 magnification and were defined as cells lining the vessel lumen that expressed both CD34 (red staining) and Ki67 (brown staining). Proliferative vessels were counted in a semi-automated manner using the method previously described by Suciu *et al.* (14).

Image acquisition and analysis were performed using a Nikon Eclipse E 600 microscope (Nikon Microscopes/Instruments Division, Vienna, Austria) and Lucia G software (Laboratory

Table I. *Characteristics of the patients for each molecular subtype of breast cancer.*

| Characteristic       | LA   | LB   | HER2 | BL   | Total |
|----------------------|------|------|------|------|-------|
| No. of cases         | 32   | 11   | 2    | 9    | 54    |
| Age, years           |      |      |      |      |       |
| Median               | 55.2 | 57.0 | 67.0 | 72.4 | 57.3  |
| Tumor diameter, n    |      |      |      |      |       |
| <2 cm                | 6    | 4    | 0    | 4    | 14    |
| ≥2 cm                | 26   | 7    | 2    | 5    | 40    |
| Grade, n             |      |      |      |      |       |
| 1                    | 2    | 0    | 1    | 0    | 3     |
| 2                    | 17   | 7    | 1    | 4    | 29    |
| 3                    | 13   | 4    | 0    | 5    | 22    |
| Stage, n             |      |      |      |      |       |
| I                    | 6    | 4    | 0    | 4    | 14    |
| II                   | 21   | 5    | 0    | 5    | 31    |
| III, IV              | 5    | 2    | 2    | 0    | 9     |
| Lymph node status, n |      |      |      |      |       |
| Negative             | 28   | 9    | 1    | 5    | 44    |
| Positive             | 3    | 2    | 1    | 4    | 10    |
| Ki67 index, n        |      |      |      |      |       |
| High (>15%)          | 1    | 8    | 2    | 8    | 19    |
| Low (<15%)           | 30   | 3    | 0    | 1    | 34    |
| MVD                  |      |      |      |      |       |
| Median               | 19   | 23.4 | 20.6 | 25.3 | 21.7  |
| MVP                  |      |      |      |      |       |
| Median               | 2.1  | 3.1  | 3    | 3.5  | 2.9   |
| ER status, n         |      |      |      |      |       |
| Positive             | 32   | 11   | 0    | 0    | 43    |
| Negative             | 0    | 0    | 2    | 9    | 11    |
| PR status, n         |      |      |      |      |       |
| Positive             | 31   | 8    | 0    | 0    | 39    |
| Negative             | 1    | 3    | 2    | 9    | 15    |

MVD: Microvessel density; MVP: microvessel proliferation; ER: estrogen receptor; PR: progesterone receptor; LA: luminal A; LB: luminal B; HER2: human epidermal growth factor receptor 2-overexpressing; BL: basal-like.

Imaging, Prague, Czech Republic) for microscopic image analysis. The entire immunohistochemical procedure was performed with Leica Bond Max (Leica Biosystems) autostainer.

**Statistical methods.** To assess the relationship between clinicopathological parameters and the immunohistochemical markers, we used Pearson, Spearman and Student *t*-test. *p*-Values of less than 0.05 were considered statistically significant. All statistical analysis was performed using the commercially available SPSS 22.0 software for Windows (IBM Corp., Armonk, NY, USA).

## Results

All cases included in the present study were histopathologically diagnosed as invasive ductal carcinoma of NST type. Most cases were graded as G2 (29 cases, 54%), followed by grade 3 (22 cases, 41%), while only three

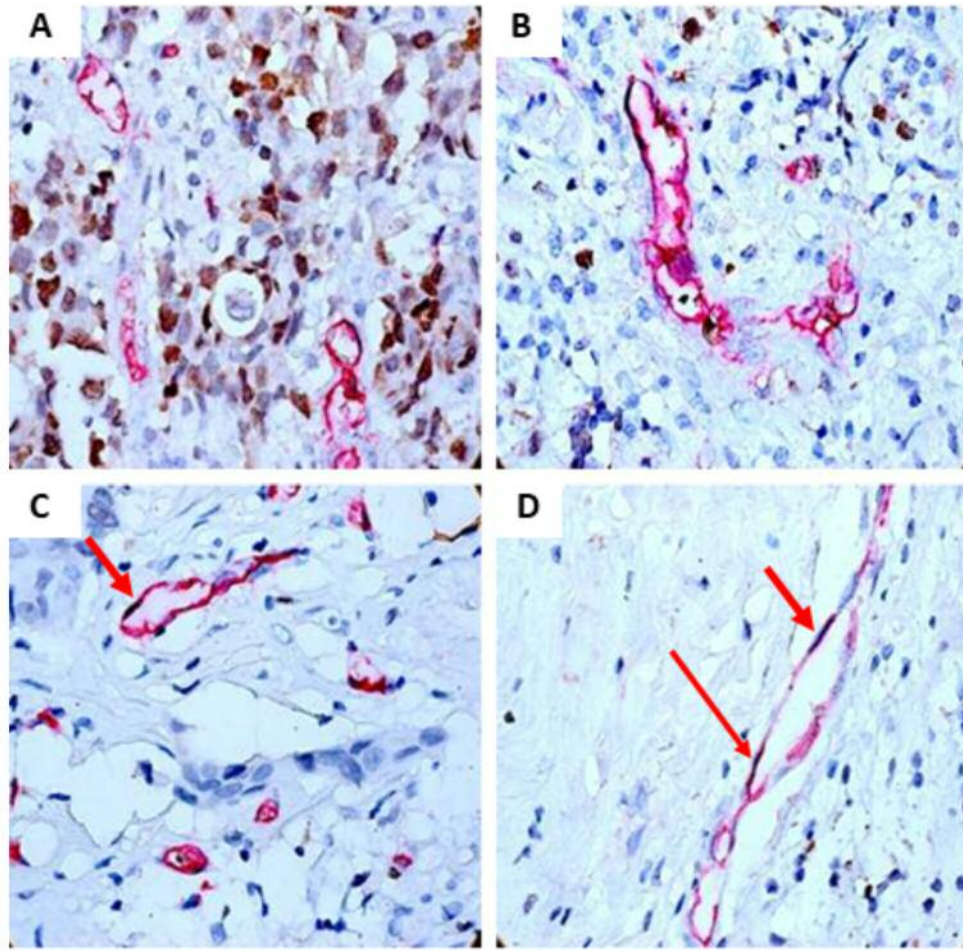


Figure 1. Angiogenesis in invasive breast carcinoma indicated by double immunohistochemistry (CD34/Ki67) showing small blood vessels (red) accompanied by endothelial cells (brown) (A, B), and small blood vessels with lumen lined by proliferating endothelial cells (arrow) (C), as well as vascular structures with a 'cord-like' aspect with proliferating endothelial cells (arrows) (D); (magnification  $\times 400$ ).

tumors were graded as G1 (5%). The median age at diagnosis was 57.3 years (range=39-85 years). Of the 54 cases included in our study, 40 tumors (74%) were larger than 2 cm, while 14 tumors (26%) were smaller than 2 cm. The median tumor diameter was 5.8 cm (range=1.3-12 cm).

By using the molecular surrogate markers, 32 out of 54 (59%) cases were classified as luminal A subtype, 11 cases (20%) were luminal B, two cases (4%) were HER2-overexpressing and nine cases (17%) had a basal-like profile. Characteristics of the patients for each molecular subtype are illustrated in Table I.

For all tumors combined, the median MVD was 21.76 vessels (range=6-68), while median MVP was 2.92 vessels (range=1-16). When each molecular subtype was evaluated separately, the highest median MVD and MVP values were recorded in the basal-like group (25.3 vessels and 3.5

vessels, respectively), while the lowest values were in luminal A tumors (19 vessels and 2.1 vessels, respectively). Notably, only MVP value reached significance in this group of patients ( $p=0.0001$ ). The highly angiogenic process that characterized basal-like tumors was sustained by the presence of small blood vessels accompanied by many endothelial cells (Figure 1A). In contrast, the other molecular subtypes were characterized by vessels with a larger lumen and with isolated endothelial cells (Figure 1B). All molecular subtypes not only contained small blood vessels with a lumen lined by proliferating endothelial cells, but also had vascular structures that tended to form a lumen, with a 'cord-like' aspect (Figure 1C and D).

For all cases combined, increased MVP was significantly correlated with negative ER status ( $p=0.010$ ) and with higher histological grade ( $p=0.002$ ). However, these associations

were not found when cases were analyzed separately based on molecular subtypes.

Neither MVD nor MVP showed any association with the other clinicopathological parameters included in this study; moreover, no correlation was found between MVD and MVP.

## Discussion

It is already known that angiogenesis has a direct impact on breast cancer development and that its level influences the prognosis of patients with this malignancy (3, 15, 16). Although the targets of anti-angiogenic agents are rational, these agents failed to add significant clinical benefits or to improve survival in patients with breast cancer (1). A possible explanation is that these anti-angiogenic agents were tested in unselected patients, without taking into account their molecular profile (1).

The concept of 'heterogeneity' in breast cancer is now widely accepted. With the advancements of new molecular techniques, it has been demonstrated that breast cancer is not a single disease but a heterogeneous one that comprise various molecular phenotypes characterized by specific behavior and prognosis (17). Molecular classification divides patients into subgroups based on their various gene expression including luminal A, luminal B, HER2 and basal-like tumors (17). This classification not only has a valuable contribution to the management of patients with this malignancy but also represents a step forward in the new era of personalized therapy.

Previous studies showed that vascular proliferation is a sensitive method for evaluation of angiogenesis, with more reliable results compared with standard MVD (18-21). MVP is a relatively new parameter of angiogenesis that measures the most active tumor vasculature, while MVD includes both preformed and newly formed vessels (6, 7). Thus, vascular proliferation reflects ongoing angiogenesis by reducing the possibility of counting vessels that are not produced by the tumor (18).

In the present study, we evaluated the relationship between angiogenesis and the molecular subtypes of invasive breast carcinoma in a series of 54 patients. We showed that basal-like tumors were associated with increased angiogenesis as estimated by MVD and MVP. However, only MVP reached significance among this group of patients ( $p=0.0001$ ). Our results are in line with previous reports that found increased vascular proliferation in basal-like breast tumors, but using different markers of vascular proliferation (18-20). The mechanism of this association is not known, although some authors suggest that vascular endothelial growth factor (VEGF), as a regulator of angiogenesis, contributes to increased vascular proliferation in basal-like tumors (18, 22). Basal-like tumors are associated with a high rate of hematogenous metastasis and with the poorest prognosis among all molecular groups. Based on our

results, we suggest that vascular proliferation can be used for stratification of patients who might benefit from therapies targeting angiogenesis.

When analyzing all the cases in this study, we found that increased MVP was significantly correlated with both negative ER status ( $p=0.010$ ) and high histological grade ( $p=0.002$ ), suggesting that vascular proliferation has a significant role in breast cancer progression. However, these associations were not found when cases were analyzed separately based on their molecular subtypes. Arnes and co-workers studied a large cohort of patients with breast cancer and demonstrated that vascular proliferation is an important prognostic factor in high-grade and ER-negative breast tumors. By contrast, MVD was not a significant prognostic indicator in their series of breast cancers, which our results are comparable with (5).

In conclusion, our study demonstrated that quantification of vessels with proliferating endothelium more accurately reflects the state of angiogenesis in breast cancer compared with standard MVD. In addition, we showed that vascular proliferation is associated with aggressive tumor features, indicating its contribution to breast cancer progression. The strong association between vascular proliferation and basal-like tumors suggests that this MVP might have an important contribution to the management of this particular group of patients. However, further studies are required to fully understand the mechanism of angiogenesis in relation to basal-like tumors and to validate the role of MVP in stratification of patients who might benefit from anti-angiogenic therapies.

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