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Research Article

# Quantitative Morphometry of the Breast Cancer Vascularity

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## Abstract

**Background:** Positive correlation of lymphangiogenesis with poor prognosis has been proven for many human malignancies, but its importance for breast cancer biology is not well recognized. Assessment of vascular network is complicated because of its irregularity and variable density within the tumor mass. Computerized mathematical analysis of tumor vascularity seems to be a promising alternative for morphometric quantitation.

**Aim:** Multifactor morphometric evaluation of vascular network in breast cancer primary tumor and regional lymph nodes comparing two antibodies: Podoplanin and CD34 to identify and assess prognostic and predictive features and factors.

**Methods:** Samples of tumors and positive and negative lymph nodes from 60 cases of breast cancer in stage pT1-pT4 were used as material for immunohistochemical assays. Morphometric parameters of vessels were estimated by CD34 and Podoplanin stains computerized analysis. Vessel characteristics assessed using CD34 staining were compared with Podoplanin estimates and correlated with T and N stage.

**Results:** The study suggests that intensity of lymphangiogenesis in breast cancer can be considered a negative prognostic factor. Presence of metastases in lymph nodes was accompanied by statistically significant increase in average density of lymphatic vessels. The examined vessel shape factors showed statistically significant differences between particular study groups. It may reflect deformation of lymphatic vessels in cases with metastasis. Also, it indicates the possibility of using the discussed morphometric features as prognostic factors.

**Conclusions:** Present study shows that Podoplanin assay provides more precise and significant estimates of vascular morphometric parameters than CD34 and therefore is recommended for further studies. The use of precise devices (tablets) in computerized image analysis of chromogene-stained vessels makes the method fast and reliable. Finally, tumor and lymph nodes vascularity characteristics might be used as prognostic and predictive factors in breast cancer combined treatment strategy planning and in treatment outcomes, respectively. Studies in this field are in progress.

## Introduction

Progress in cancer biology has improved our knowledge on natural tumor growth, vasculogenesis and metastatic potential. During the last 20 years a system of many factors regulating the process of neovasculogenesis (VEGFs, Prox-1, FGF, HIF-1, PDGF, Ang-1, Ang-2, Lox, Met, TGF, IGF, MMP-2, uPAR) and their receptors and relationships have been recognized. Methods of immunohistochemical identification of the pathologic vessels (CD34, CD31, factor VIII, VEGFs, Podoplanin, LYVE-1, Prox-1, Ang-1, Ang-2) are widely used in practice. However, an impact of angio- and lymphangiogenesis on kinetics and cancer dissemination is still open to discussion [1-7]. Enormous pathological and biological heterogeneity of solid tumors recruited to the analyses raises more questions and doubts than reliable answers. There is general lack of common standards of the tissue specimens used for immunohistochemical tests (either tumor borders or its central part). In contrast to melanoma and rectal, head and neck, ovarian, thyroid and lung cancer in which lymphangiogenesis correlates with poor prognosis, importance of this process for development and progression of breast cancer is not well recognized. One of the crucial points is which quantitative method allows to count

vascular density most precisely [8,9]. It is not easy to identify and assess vascular network because of its irregularity and altered density within the tumor mass. Such analyses may provide misleading results (lack of lymphangiogenesis, no correlation with kinetic of tumor spread whereas these mechanisms exist). Apart from vascular density, other non-parametric factors as vessel shape, lengthening, wall structure and filling by blood cells seem to play important role and should also be evaluated. Computerized mathematical analysis of tumor vascularity images (fractionary analysis, three-dimensional box model) seems to be an promising option for vessel morphometric quantitation [10,11]. Therefore, goal of the present study is identification and quantitation of morphometric characteristics of tumor and lymph nodes vascularity and its predictive value for treatment of breast cancer patients.

## Material and methods

### Material

Material for the present study are paraffin-embedded tumor specimens of 60 consecutive cases of breast cancer treated by mastectomy and regional lymphadenectomy in the period 2001-2003. Average age of the patients was 57 years (35 to 83 years). Tumor diameter ranged from 0,7 cm to 15 cm and T stage was: 29 cases (48%) with pT1, 29 with pT2 (40%), 1 with pT3 (2%) and 6 cases (10%) with pT4. There were 13 tumors with Grade 1 (G1), 36 - G2 (60%) and 11 with G3. Number of evaluated regional lymph nodes ranged from 11 to 24 per patient. In the group of 22 patients (37%) lymph nodes were positive. Tumor and lymph node specimens included in the analysis were subdivided into the following three groups:

Group I - tumors with negative nodes T-N(0) versus tumors with positive nodes T-N(+).

Group II - nodes without metastases N(0) versus metastatic nodes N(+).

Group III - individual node without meta in cases without meta in the remaining excised nodes

N(-) : N(0) versus nodes without meta in cases with meta in the remaining nodes N(-) : N(+).

### Methods

At first, tumors and lymph nodes were evaluated histopathologically. Tumor specimens included tumor borders and margins of surrounding normal tissues. From lymph node specimens the largest nodes were selected, one positive and one negative. From the group N(-) two largest nodes were chosen. Then immunohistochemical assays using monoclonal antibodies: CD34 (Mouse Monoclonal, clone QBEnd 10, Dako Denmark) and Podoplanin (Mouse Monoclonal, clone 18H5, Abcam) were carried out. Acquisition of the digital high definition microscopic images under 200x magnification was performed using DFC490 camera. Series of digital images were prepared for computerized analysis using graphic high definition tablet (Wascom CTH-661) with manual-edited correction. The immunostained vessels (Figure 1A) were contoured (Figure 1B) on original images. Then the binary masks were generated to prepare morphometric profiles of the individual vessels (Figure 1C).

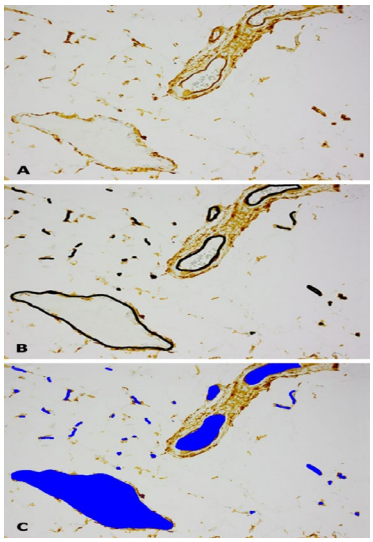


**Figure 1:** Steps of computer assisted image analysis. **A:** Original image of immunostaining; **B:** Manual completion of vessels' outlines; **C:** Mask generation and segmentation of the immunostained image.

Following vessel parameters were measured:

a. VA - area in $\mu m^2$ .

b. VG - circumference in µm.

c. VR - roundness factor defined as: $VR=VG^2/(4\ \pi\ *VA)$ [$\mu m^2/\ \mu m^2$].

d. VL - lengthening - using equation: $VL=La*Lb$ [$\mu m^2$] where La is the longest and Lb - the shortest dimension of the vessel.

e. FI - vessel filler index defined as $FI=\sqrt{VA}*VC$ [$\mu m^2$] where VC is vessel contour area.

Distribution of the morphometric parameters was evaluated using Kolmogorow-Smirnow test with significance of $p\leq0.05$. Because results did not show normal distribution, non-parametric statistics (U-Mann-Whitney's test) and Statistica version 8.0 (Statsoft Inc.) program were used.

## Results

All together 9996 CD34 profiles and 1627 Podoplanin profiles were examined (Table 1).

| Group | Subgroup | Number of profiles examined CD34 | Number of examined profiles Podoplanin | Accordance to the normal distribution |
|---|---|---|---|---|
| **I** | T-N(-) | 2379 | 469 | Shapiro-Wilk normality test negative ($p<0.05$) |
| | T-N(+) | 1585 | 267 | |
| **II** | N(-) | 1585 | 455 | |
| | N(+) | 1468 | 128 | |
| **III** | N(-):N(-) | 1626 | 201 | |
| | N(-):N(+) | 1413 | 107 | |

**Table 1:** Definition of the study groups and numbers of analyzed morphometric profiles in each group.

### Vessels' area and circumference

Results of area and circumference measurements using CD34 and Podoplanin stains are shown in Table 2 and 3.

| Group | | Mean area [$\mu m^2$] | Statistical significance (p) |
|---|---|---|---|
| CD 34 assays | | | |
| I | T-N(-) | 225.9 | 0.09 |
| | T-N(+) | 284.8 | |
| II | N(-) | 395.5 | 0.04 |
| | N(+) | 289.0 | |
| III | N(-):N(-) | 241.7 | 0.04 |
| | N(-):N(+) | 282.9 | |
| Podoplanin assays | | | |
| I | T-N(-) | 710.5 | 0.001 |
| | T-N(+) | 892.0 | |
| II | N(-) | 623.4 | 0.0001 |
| | N(+) | 2191.4 | |
| III | N(-):N(-) | 1235.5 | 0.19 |
| | N(-):N(+) | 1238.5 | |

**Table 2:** Area of the vessels stained with CD34 and Podoplanin.

| Group | | Mean circumference [µm] | Statistical significance (p) |
|---|---|---|---|
| CD 34 assays | | | |
| I | T-N(-)<br>T-N(+) | 68.5<br>64.9 | 0.36 |
| II | N(-)<br>N(+) | 75.4<br>59.3 | 0.001 |
| III | N(-):N(-)<br>N(-):N(+) | 69.4<br>61.1 | 0.28 |
| Podoplanin assays | | | |
| I | T-N(-)<br>T-N(+) | 136.7<br>99.1 | 0.05 |
| II | N(-)<br>N(+) | 128.9<br>64.8 | 0.01 |
| III | N(-):N(-)<br>N(-):N(+) | 120.0<br>76.4 | 0.048 |

**Table 3:** Circumference of the vessels stained with CD34 and Podoplanin.

Area of the lymphatic vessels stained with Podoplanin was significantly much larger in both tumors with positive lymph nodes and positive nodes (group I and II) per se than in tumors in stage N(0) and nodes without meta (Table 2). However, no difference in the areas of lymphatic vessels in negative nodes accompanied with other positive or negative nodes (group III) was noted (Table 4,5).

| Profiles / Parameters | CD34 | | | | PDPL | | | |
|---|---|---|---|---|---|---|---|---|
| | T-N(-) | T-N(+) | N(-) | N(+) | T-N(-) | T-N(+) | N(-) | N(+) |
| Density | no change | | | | ↑ | ↑ | ↑ | ↑ |
| Area [µm²] | | | | | ↑ | ↑ | ↑ | ↑ |
| Circumference [µm] | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ |
| Roundness [µm²/ µm²] | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ |
| Lenghtening [µm²] | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ |
| Filler index [µm²] | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ |

T - tumour, N - node; (-) no meta, (+) with meta

**Table 4:** Characteristics of lymphatic vessels profiling by CD34 or Podoplanin (PDPL) assays.

| Profiles / Parameters | Tumour N(-) vs. Tumour N(+) | | | | Lymph node(-) vs. Lymph node(+) | | | |
|---|---|---|---|---|---|---|---|---|
| | CD34 | | PDPL | | CD34 | | PDPL | |
| Area [µm²] | ↑ | | ↑ | | ↑ | | ↑ | ↑ |
| Circumference [µm] | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ |
| Roundness [µm²/ µm²] | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ |
| Lenghtening [µm²] | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ |
| Filler index [µm²] | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ |

(-) no meta, (+) with meta

**Table 5:** Characteristics of lymphatic vessels profiling by CD34 or Podoplanin (PDPL) assays.

Although differences in average values of the circumference stained by Podoplanin were statistically significant in each of the analyzed groups, average girths for tumors with positive nodes and also positive nodes per se were shorter, but it was not very well pronounced compared with N(0) tumors and negative nodes. Similar tendency was noted in the results for the CD34 staining (Table 3).

### Vessel roundness and lengthening

Results of the roundness estimates using CD34 and Podoplanin are presented in Figure 2. In all analyzed subgroups similar significant tendency of lower roundness estimates for tumor with positive nodes, metastatic nodes and nodes free of metastases but accompanied with other positive nodes was noted compared with N0 tumors, individual negative nodes and negative nodes accompanied with nodes free of metastases. Although average values differ significantly, wide range of 95% confidence limits suggests careful interpretation.

| | | | | | |
|---|---|---|---|---|---|
| T-N(0) | T-N(+) | 2.79 | 2.62 | 2.22 | 1.1 |
| N-N(0) | N-N(+) | 1.9 | 1.34 | 2.04 | 0.95 |
| N(-)-N(0) | N(-)-N(+) | 2.76 | 2.63 | 2.21 | 1.05 |
| | | 1.86 | 1.92 | 1.88 | 0.88 |
| | | 3.29 | 4.68 | 2.17 | 1 |
| | | 1.91 | 1.87 | 1.96 | 0.95 |
| | | | | | |
| T-N(0) | T-N(+) | 3.34 | 2.73 | 2.8 | 1.72 |
| N-N(0) | N-N(+) | 2 | 1.58 | 2.02 | 1.17 |
| N(-)-N(0) | N(-)-N(+) | 3.18 | 2.58 | 2.77 | 1.66 |
| | | 2.32 | 1.99 | 2.19 | 1.25 |
| | | 2.87 | 2.16 | 2.66 | 1.69 |
| | | 2.35 | 1.93 | 2.04 | 0.89 |

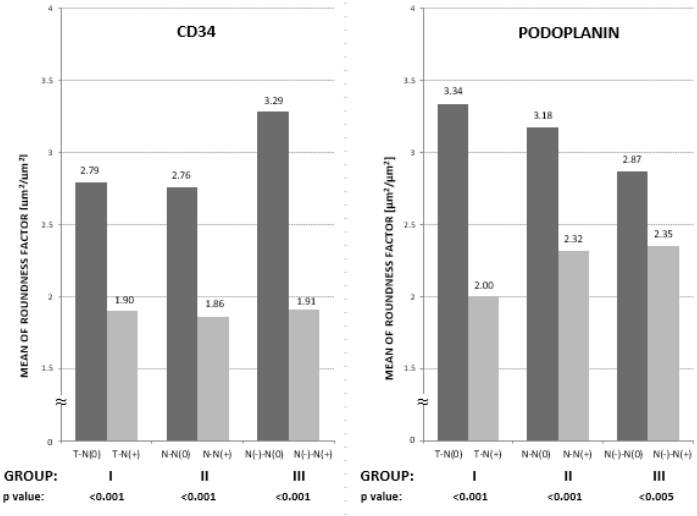


**Figure 2:** Histograms of mean roundness values and significance values (p).

Differences in vessel lengthening were even less pronounced although significant tendency as for vessel roundness was noted (Figures 3 and 4). Once again wide range of 95% confidence limits in all subgroups shows large variation of the individual estimates.

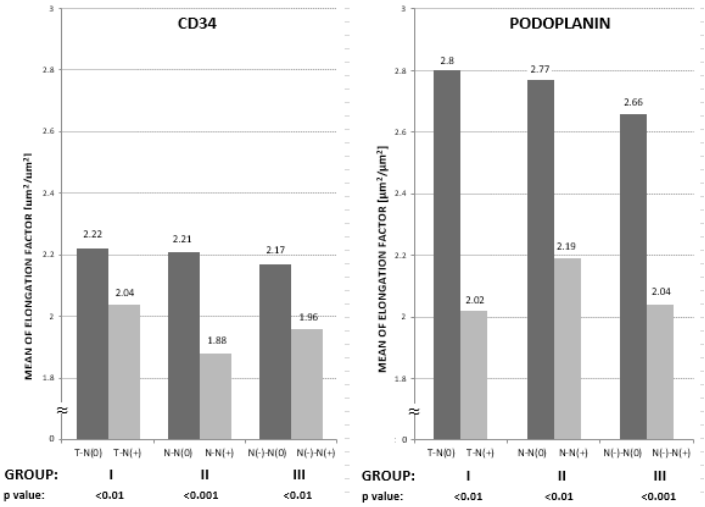


**Figure 3:** Histograms of mean elongation factor values and significance values (p).

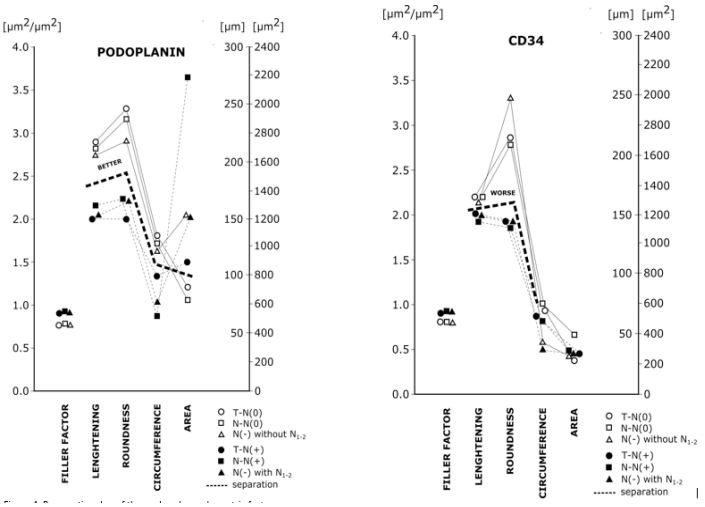


**Figure 4:** Prognostic value of the analyzed morphometric factors.

### Filler Index (FI)

There were no pronounced differences in filler indexes between analyzed subgroups because estimates values were within very narrow range from 0.76 to 0.88.

## Summary

Among all tests carried out using CD34 assays average area of the lymphatic vessels was larger in tumors with positive nodes, metastatic nodes versus those free of metastases. Podoplanin assay seems to be more predicting for vessels area in positive nodes which was more than 2.5 times larger than nodes free of meta (Table 3, group II). Regarding nodes, circumferences of

vessels were smaller in positive nodes and tumors with N(+) than in negative nodes and tumors T-N(0), but using Podoplanin assay these differences were much better pronounced. Lymphatic vessels' roundness was higher in N(0) tumors and negative nodes similarly estimated by CD34 and Podoplanin assays (Figure 2). However, for both series of the estimates 95% confidence limits were rather wide. The results suggest that lengthening and filler indexes do not differ very much between negative and positive nodes and tumors (Figure 4).

## Discussion

During the recent years, majority of the published studies have shown significant correlation of lymphangiogenesis with tumor progression and poor prognosis [12], in cancers of colon and rectum [13], head and neck [14,15], cervix [16], stomach [17], thyroid gland [18] and ovary [19], and also melanoma [20,21]. In melanomas, colorectal and thyroid cancers higher lymphatic vessel density occur at the peripheral part of the tumor, whereas in head and neck cancer higher density has been found mainly in the center of the tumors. In cervical cancer higher lymphatic density may occur at the peripheral or central part of the tumor. Results concerning prostate cancer are controversial [22-25]. Concerning lymphangiogenesis in breast cancer, whether peripheral or central part of the tumor is more representative is still widely discussed [26,27]. Some authors point out that in breast cancers angiogenesis is more intensive than lymphangiogenesis [28]. There are even suggestions that lymphangiogenesis in breast cancers does not exist, and the identified vessels might be in fact persistent ones, which already existed in the normal tissue before the tumor has developed [29,30]. Therefore, it is not as easy to evaluate lymphatic network in breast cancer as it may seem, and simple morphometric methods are probably not effective enough. To the present study only invasive ductal carcinomas have been selected to get as biologically homogenous clinical data as possible.

Deliberately, our study has focused on the peripheral part of the tumor and lymph nodes and surrounding normal tissues. It is siginficant that our morphometric analyses concentrated on circumnodal and circumtumoral parts in cases of negative versus positive nodes. Till now, the results of simultaneous analysis of tumoral and nodal lymphangiogenesis have not been published yet. Based on the "First international consensus on the methodology of lymphangiogenesis quantification in solid human tumours", CD34 and Podoplanin assays were used. Although Chalkley's morphometric method is recommended due to its repeatability and reliability, it has some limitations [9,28,31]. Adipose tissue strand surrounding lymph node is relatively narrow to be covered by 25-point net diagram. Therefore we used Weidner method and vessels were quantified in three arbitrarily selected hot-spots [31]. In the present study computerized fractal analysis of vascular network images stained by CD34 or Podoplanin was used [32]. This method provides characteristics and measurements of even the most complex vascular networks using simple mathematical equations, because fractals represent many levels of vascular branches and provide faithful copies of complicated vascular systems.

It is not easy to count the vessels because of some uncertainties regarding their size (new vessels are usually very small), irregular shape and sinusoidal course [24,25,33]. Graphical tables were used to select hot-spot areas, contour vessel margins and close incomplete profiles. Computer analyses of such images allow to define vessel shape and estimate its average area, circumference, roundness, lengthening and filler parameters. Results of our analyses are shown in Tables 2 and 3, Figures 2-4 and are summarized in Tables 4 and 5 which generally show that Podoplanin staining seems to be more effective in estimating morphometric parameters characterizing lymphangiogenesis in breast tumors and lymph nodes than CD34. Vessel density was higher in both positive nodes and tumors with positive nodes stained by Podoplanin whereas there was no difference when CD34 was used.

Harwell et al pointed out that changes in lymphangiogenesis might be interpreted as a prerequisite for the development of metastasis within the lymph nodes [34]. However, Tretiakova et al. suggest that vessel density should not be considered as a key parameter, in contrary to the vessel area, circumference and wall thickness [24]. Similarly, Munaron at al. and Korkolopoulou at al. noted that shape (roundness) and size of the area are more important than vessel density and that roundness seems to be a single most powerful predictive factor for tumor progression [35,36]. Present results (Table 5) support observations of other authors that Podoplanin staining demonstrates larger circumference, roundness and lengthening of lymphatic vessels in negative nodes and tumors with negative nodes than in positive nodes and tumors with positive nodes. It was not so explicit when CD34 staining was used. Vessel circumference was smaller in tumors in stage T-N(+), positive nodes (Table 4) but also in nodes without meta but accompanied by positive nodes (Table 3, group III). Similar tendency was noted when roundness and lengthening were evaluated. The results illustrating changes in the network of lymphatic vessels in positive nodes and also in negative nodes accompanied with positive nodes support Harwell's suggestion that it might be a predictive factor of the premetastatic phase.

Higher number and larger diameter of the lymphatic vessels which are lengthened and more sinusoidal with increased permeability characterize chaotic and disorganized pre- or metastatic nature of lymphatic network [4,24,25,37]. Such structure favors cancer cells penetration through the vessel wall to circulate in lymphatic network and develop regional and distant metastases [25,37]. However, relatively wide range of 95% confidence limits noted in the present analyses need careful interpretation and confrontation with therapeutic strategy and long term treatment

outcomes, which is in progress.

## Conclusion

Results of the present study suggest that morphometric quantitative estimates of the parameters of lymphatic network in breast cancer and regional lymph nodes (size of the area, circumference, roundness, lengthening and density) provide important information supplementing clinical diagnostic procedures. It might be, at least for some selected cases, a prerequisite for pre-metastatic phase in the regional lymph nodes and a predictor of metastatic potential. Present study shows that Podoplanin staining is a more powerful, precise and significant assay than CD34 staining.

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