

# Microvessels in Epithelial Ovarian Tumors: High Microvessel Density Is a Significant Feature of Malignant Ovarian Tumors

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**Abstract.** *Background/Aim:* Examine features of blood and lymphatic vessels in ovarian tumors and their significance to prognosis of ovarian cancer. *Patients and Methods:* A total of 139 women with epithelial ovarian tumors were included: 86 malignant, 17 borderline and 36 benign. Density, percentage, mean size and number of blood microvessels in tumors were measured by immunohistochemistry with antibodies against CD34 and CD105. Lymphatic vessel density was assayed using the D2-40 antibody against podoplanin. *Results:* Angiogenesis was most profuse in malignant tumors. Small size of lymph vessels predicted 26% shorter 5-year survival of ovarian cancer patients. Further, high percentage of lymphatic vessels in tumors was associated with lymph node metastasis, and high density with cancer recurrence. Lower number of microvessels, as assessed by CD34 staining, predicted shorter progression-free survival. Additionally, the large size of microvessels assessed by CD34 and the high number of vessels assessed by CD105 were related to residual tumor >1 cm at primary surgery and also, large vessel size was associated with stage III, as assessed by CD105 staining. *Conclusion:* CD34 and CD105 define different characteristics of microvessels. Parameters of lymph vessels may predict the prognosis of ovarian cancer.

Ovarian cancer has the worst prognosis among all gynecological malignancies. Due to its treacherous nature, it is often diagnosed in advanced stage (70%). In spite of the primary treatment: cytoreductive ultraradical surgery and chemotherapy with carboplatin and paclitaxel, disseminated or recurrent ovarian cancer can rarely be completely cured, the 5-year survival still being 27% (1, 2).

Targeted treatment strategies have ushered in a new era in cancer therapy. The antiangiogenic monoclonal vascular endothelial growth factor (VEGF) antibody bevacizumab, has been part of the standard care of stage IV and III suboptimally debulked primary cancer, as well as of recurrent ovarian cancer (3, 4). In addition, an antiangiogenic approach has been combined poly (ADP-ribose) polymerase (PARP) inhibition and immunological checkpoint inhibitors in ongoing trials to boost the treatment effect (5, 6). However, optimal method for selecting patients for the antiangiogenic therapy is still obscure; it includes biomarkers and the knowledge of the diagnostic characteristics of the disease, prognostic means and follow up of treatment efficacy.

Due to neoangiogenesis, the structure of the vessels in malignant tumors differs from that of the normal ones. In malignant tumors, microvessels are curvier and organized more irregularly (7). Further, their walls are more fragile since the endothelial cells are weakly connected to each other and to the pericyte layer (8), and the basal membrane has also only loose contact with the pericytes (9).

Hypoxia activates angiogenesis in tumors. VEGF has an important role in the growth of endothelial cells. VEGF-C and -D, activate vascular endothelial growth factor receptor 3 (VEGFR3), and are in turn vital for the development of lymph vessels (10). In several studies, VEGF has been shown to be over-expressed in many malignancies like

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**Key Words:** Ovarian cancer, immunohistochemistry, angiogenesis, lymphangiogenesis, CD105, CD34.

breast, ovary, endometrium and bladder tumors (11); however, controversial results have also been described (12).

Microvessel density describes the intensity of angiogenesis in the tumors. Several studies have shown that high microvessel density, VEGF levels and the expression of VEGF receptors are associated with poor prognosis (13). On the other hand, there are contradictory results regarding the value of high microvessel density and its relation to prognosis. In some studies, it has been associated with a better prognosis (14, 15). It has been suggested that increased blood supply to the tumors enhances the access of the cytoreductive agents and oxygen to the tumor. There are also studies where a correlation between microvessel density and prognosis was not found (16).

Antibodies against cluster of differentiation 31 (CD31) and CD34 are used in immunohistochemical expression studies of the endothelium. Glycoprotein CD34 is expressed on the endoepithelial surface and on the cell membranes, connecting the layers of endothelial cells, and is also expressed in immature blood cells (17).

Endoglin, also known as CD105, is a cell membrane homodimeric glycoprotein and a part of the transforming growth factor (TGF- $\beta$ ) receptor complex. Endoglin is expressed by rapidly dividing endothelial cells and is vital for angiogenesis. Endoglin has been related to newly formed blood vessels, while CD34 is expressed in both old and newly formed vessels (17, 18). High microvessel density, as measured by endoglin, has been related to a disseminated cancer and poor prognosis of breast, prostate, cervix, gastrointestinal and head and neck cancers (19-22). Besides this, endoglin has been shown to activate signaling pathways that prevent endothelial cell apoptosis (18).

Mechanisms of lymphangiogenesis are less known and fewer specific antibodies are available to study the characteristics of lymphatic vessels. Podoplanin is expressed in the endothelium of lymph vessels, and is used in lymph vessel staining. The monoclonal antibody D2-40 adheres to podoplanin and is used for the measurement of lymph vessel density. Its high expression has been related to cancer aggressiveness (23).

In this study, we compared the two staining methods, CD34 and endoglin, for their accuracy in assessing angiogenesis in ovarian cancer and evaluated the differences in microvessels according to the type of tumor. Additionally, the significance of lymph vessels was examined in relation to the prognosis of ovarian cancer patients.

## Patients and Methods

*Patient and data collection.* This study included 139 women, who were diagnosed and operated for epithelial ovarian tumors at Kuopio University Hospital between 1999 and 2007. Of these women, 86 had malignant epithelial ovarian cancer, 17 had borderline and 36

had benign tumors (Tables I and II). The histological subtypes and the grades of tumors were evaluated according to the World Health Organization (WHO 2003) Classification of Tumors (24). Tumor tissue samples were collected during primary surgery and samples were analyzed using immunohistochemistry. The follow-up time ended in November 2019. Exclusion criteria included: nonepithelial neoplasms, the need for neoadjuvant therapy, and unresectable cancer. Staging followed the criteria of the International Federation of Gynecology and Obstetrics (FIGO 1988). Most included cancer patients were treated with platinum-based chemotherapy after operation. In total, 42% (n=36) received paclitaxel and carboplatin as their primary adjuvant treatment. 30% (n=26) started chemotherapy with the combination of paclitaxel and carboplatin, but were changed to single carboplatin, because of side effects. Furthermore, 13% (n=11) had single carboplatin. Three women were treated with cisplatin combined with cyclophosphamide, two women with gemcitabine and carboplatin, and one with cisplatin and paclitaxel. For seven patients, either chemotherapy was not administered because of stage IA cancer or information on the treatment was not available. None of the patients received bevacizumab as part of their first line treatment (12). The study was approved by the Ethical Committee of the Kuopio University Hospital, Finland (26/99). Written informed consent was obtained from all patients participating the study. This study adhered to the principles of the Declaration of Helsinki.

*Immunohistochemistry.* Tissue samples were embedded in paraffin and cut into 5- $\mu$ m-thick sections. Next, the sections were processed for microvessel staining with anti-CD34 antibody (mouse monoclonal antibody CD34, Vectastain Elite kit, Dako, Glostrup, Denmark, 1:500) and anti-CD105 antibody (mouse monoclonal antibody CD105, Biocare Mach 1 kit, 1:90). Lymph vessel staining was performed using an anti-D2-40 antibody (mouse monoclonal antibody D2-40, Vectastain Elite kit, Dako, Glostrup, Denmark, 1:200). Immunostained samples were evaluated with use of the Olympus AX70 microscope (Olympus Optical, Tokyo, Japan). Three hot spots with the highest density of microvessels or lymph vessels per sample were chosen and photographed with  $\times 200$  magnification (25-27). Necrotic areas were not counted. Images were analyzed with the analySIS-program (Olympus, Soft Imaging System, GmbH, Münster, North Rhine-Westphalia, Germany) that measures microvessel density (MVD, vessels/mm<sup>2</sup>), lymphatic vessel density (LVD, vessels/mm<sup>2</sup>), the mean size/ area of the vessels ( $\mu$ m<sup>2</sup>), the percentage of vessels (%) in the tumor and the number of vessels (N).

Immunostaining of VEGFs and VEGF receptors was processed and evaluated as in our previously published study (12).

*Statistical analysis.* Statistical analyses were performed using Statistical Package for Social Sciences (SPSS for Windows version 24, 1989-2016, SPSS Inc., Chicago, IL, USA). Kruskal–Wallis test was performed followed by the Mann–Whitney test for multiple comparisons of continuous variables. For analyses of the association of the clinicopathological factors and survival, each variable was dichotomized into low and high groups using the median values as a cut-off. The chi-squared test was performed to analyze frequency tables for categorical variables. The impact of the variables on the progression-free survival and overall survival was defined with the Kaplan–Meier method. The correlations were analyzed with the Spearman's test (12).

Table I. Characteristics of 139 studied women with ovarian tumors

	Benign tumors	Borderline tumors	Malign tumors
Total	36 (100)	17 (100)	86 (100)
Age by the time of diagnosis, median (years)	56 (16-92)	66 (20-92)	58 (29-88)
Histological subtype			
Serous	21 (58)	11 (65)	51 (59)
Mucinous	15 (42)	6 (35)	11 (13)
Endometrioid cancer			15 (17)
Clear cell cancer			5 (6)
Other			4 (5)

Values are N (%) or median (range).

## Results

*Association of microvessel staining with ovarian tumor type.* There were significant differences in the association between microvessel parameters as assessed by CD34-staining and the type of ovarian tumor (Figure 1, Table III). In malignant tumors, the microvessel density, mean area, the percentage, and the number of microvessels assessed by CD34 were significantly higher compared to the respective values of the benign tumors ( $p<0.001$ ;  $p=0.044$ ) (Figure 2A-D). In addition, in malignant tumors, microvessel density and the number of vessels were higher than those in borderline tumors ( $p=0.010$ ) (Figure 2B and D). The mean area and the percentage of microvessels of borderline tumors were significantly larger than those in benign tumors ( $p=0.001$ ;  $p<0.001$ ) (Figure 2A and C).

Endoglin staining showed, that the microvessel density in ovarian cancer was significantly higher (31%) than that in borderline tumors ( $p=0.046$ ), and in benign tumors, the difference was even higher (70%) ( $p<0.001$ ) (Figure 2F). Additionally, there were 35% more vessels in malignancies compared to borderline tumors ( $p=0.036$ ) and double more compared to benign tumors as assessed by endoglin staining ( $p<0.001$ ) (Figure 2H) (Table III). Mean size and percentage of microvessels in tumors assayed by endoglin were not different between malignant, borderline and benign tumors (Figure 2E and G).

Lymph vessel features were not related to tumor type (Figure 2I-L); there were no significant differences in lymph vessel density, percentage, size or number of lymph vessels between different tumor histologies.

*Relation of clinicopathological data to the microvessels in ovarian cancer.* The higher percentage of microvessels observed by CD34 staining ( $p=0.001$ ) and the greater number of vessels seen by endoglin ( $p=0.012$ ) staining in ovarian malignancies were associated significantly with a larger residual tumor (>1 cm) after primary surgery (Table IV).

Table II. Clinical characteristics of the 86 ovarian cancer patients.

Characteristic	Ovarian carcinoma N (%)
Histologic subtype	
Serous	51 (59)
High grade	45
Low grade	6
Endometrioid	15 (17)
High grade	13
Low grade	2
Ca12-5, kU/l	363 (5-10100)
FIGO stage	
I	12 (13)
II	10 (12)
III	46 (53)
IV	18 (22)
Histological grade*	
1	9 (12)
2	25 (33)
3	41 (55)
Ascites	60 (70)
No ascites	16 (19)
Unknown	10 (11)
Metastasis in lymph nodes	
Yes	5 (6)
No	22 (26)
Unknown	59 (69)
Residual tumor at primary surgery	
None	40 (47)
≤1 cm	8 (9)
>1 cm	38 (44)
Chemotherapy response	
Complete response	57 (66)
Partial response	4 (5)
Stable disease	2 (2)
Progressive disease	5 (6)
No chemotherapy	5 (6)
No data	13 (15)
Tumor recurrence	49 (57)
No recurrence	26 (30)
No data on recurrence	11 (13)
Patient status	
Dead from ovarian cancer	52 (60)
Dead for other/unknown reason	6 (7)
Alive	28 (33)
Median follow-up, months	66 (0-198)

Values are presented as N (%) or as median (range). \*Mucinous tumors were not graded.

The high percentage of lymph vessels in ovarian cancer predicted significantly greater probability of lymph node metastasis ( $p=0.008$ ). The higher lymph vessel density ( $p=0.013$ ) and number of lymph vessels in the tumor ( $p=0.042$ ) were significantly associated with ovarian cancer recurrence. In addition, features of active lymphangiogenesis including high density and number of lymphatics, were related to high-grade cancer ( $p=0.002$ ;  $p=0.035$ ), and high

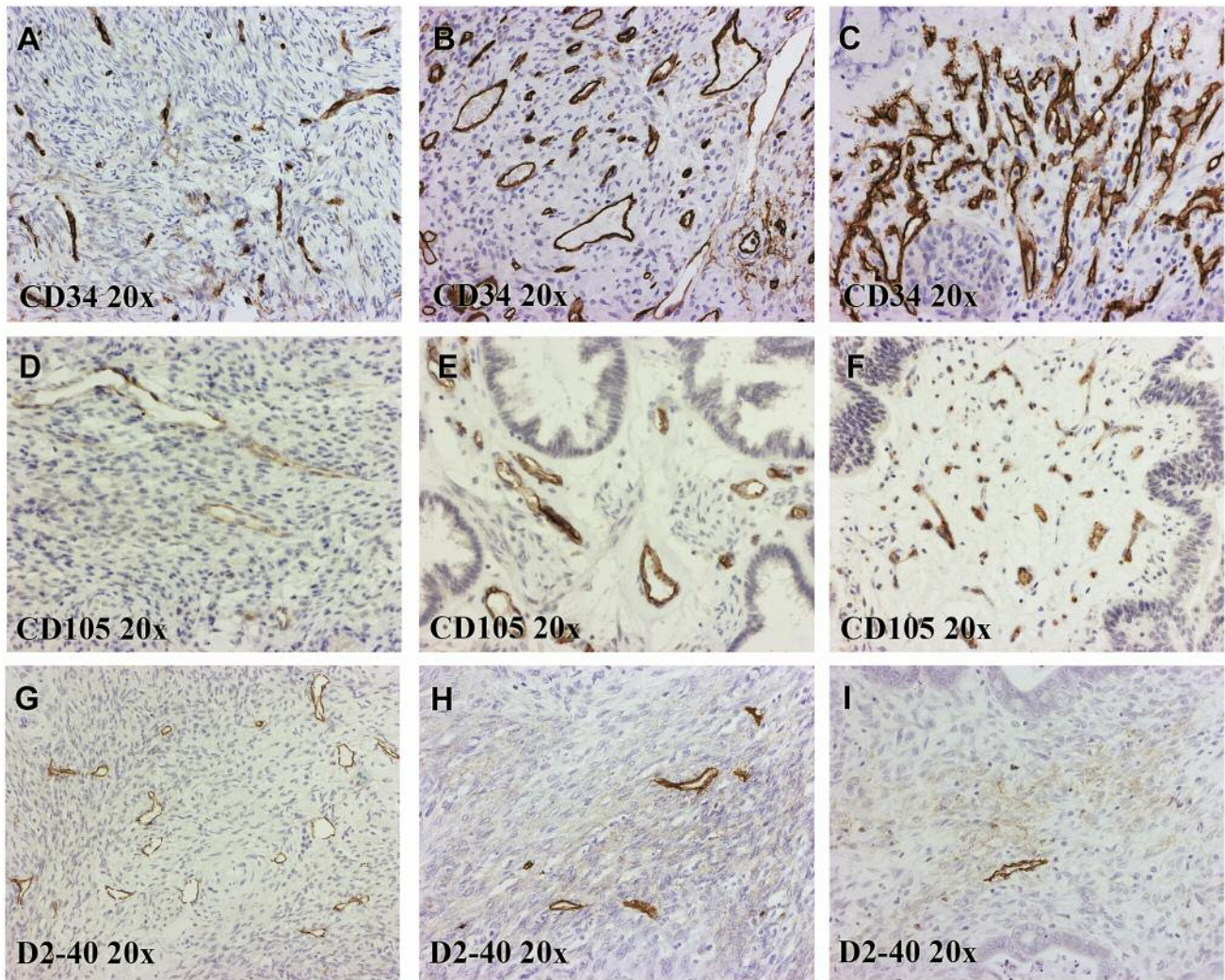


Figure 1. Immunohistochemistry of micro- and lymph vessels. By CD34 staining more microvessels were found in malignant tumors (C) compared to the borderline (B) or benign tumors (A). Also, by endoglin (CD105) staining, microvessels were more profuse in cancer (F) than in borderline (E) or benign tumors (D). The percentage of lymph vessels in the tumor was higher in serous cancer (G) compared to the malignant tumors of other histology (H, I; mucinous carcinoma, endometrioid cancer).

percentage of lymph vessels to serous histologic type ( $p=0.010$ ) (Table IV).

The results were comparable when analyzing categorical variables by Pearson's chi-squared test and as continuous variables by Kruskal–Wallis test.

**Progression-free survival and overall survival of the ovarian cancer patients.** The median follow-up time was 66 months (range=0-198 months). At the end of follow-up, 58 (67%) of the ovarian cancer patients had died. Progression-free survival analysis included 75 patients, of whom 65% experienced recurrence. The median progression-free survival was  $16\pm12$  months.

The small size of the lymph vessels predicted shorter overall survival of the ovarian cancer patients (medians: 42 vs. 77 months;  $p=0.035$ ) (Figure 3).

The higher number of microvessels assessed by CD34 staining was associated with longer progression-free survival ( $p=0.020$ ).

In endoglin staining, the properties of microvessels did not reach significance in association with cancer prognosis. However, the tendency was clear: the more vessels in the tumor, the shorter the overall survival was.

**Correlation between density, percentage, size and number of microvessels, with VEGF and VEGF receptor expression in**



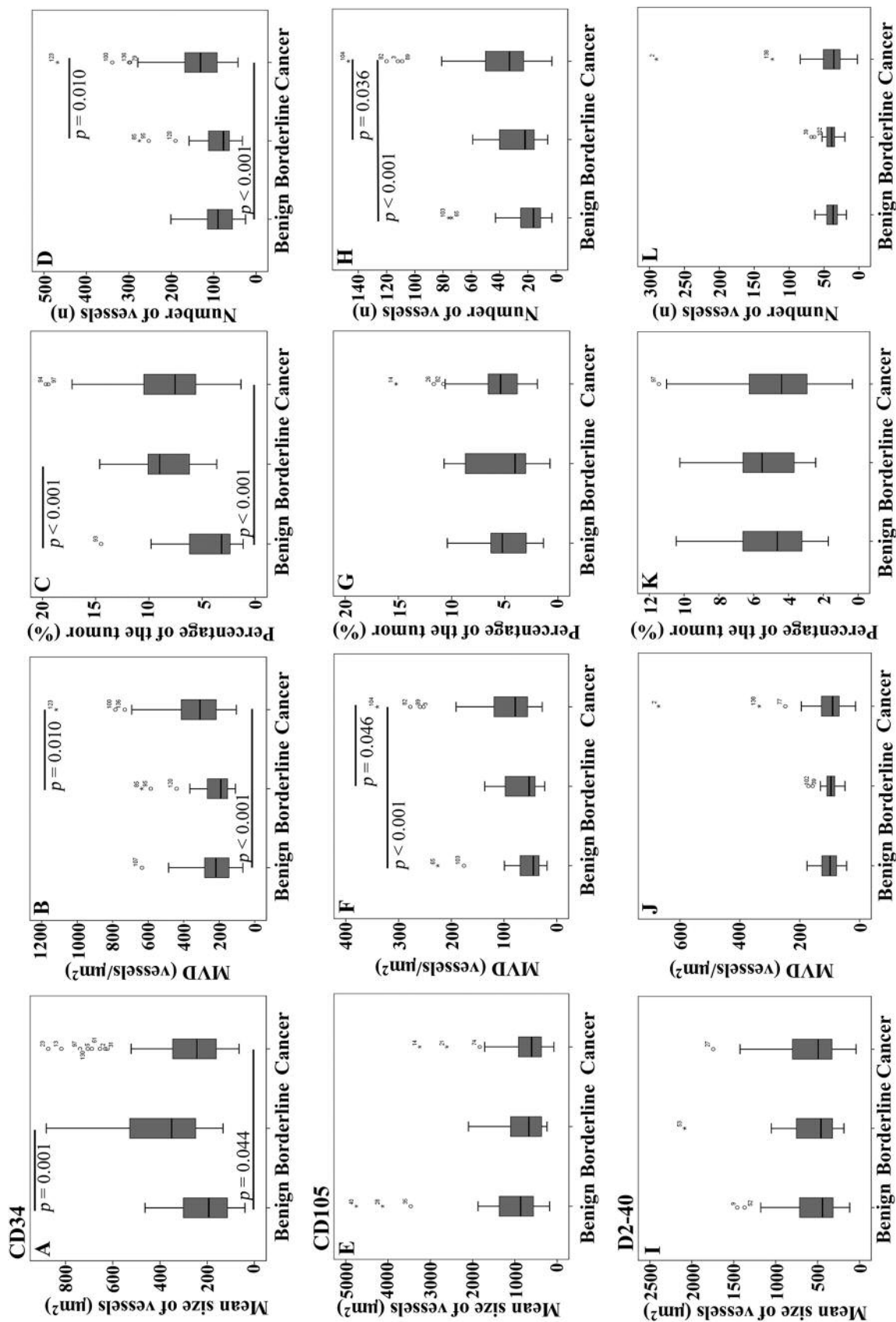


Figure 2. Features of micro- and lymph vessels according to the type of ovarian tumor. Vessels were larger in malignant and borderline tumors compared to the benign tumors as assayed by CD34 staining (A). Microvessel density (MVD) and the percentage of vessels in the tumor were also higher in malignant and borderline tumors than in benign tumors as assessed by CD34 staining (B, C). By endoglin (CD105) staining, malignant tumors had higher microvessel density than borderline and benign tumors (F). The number of vessels were more profuse in cancer compared to the borderline and benign tumors as assessed by endoglin staining (H). Mean size and percentage with the type of tumor did not correlate with the type of tumor as assayed by endoglin (E, G). Lymph node staining, did not reveal significant differences between the types of tumor (I, J, K, L).

Table III. Features of microvessels in relation to the type of ovarian tumor.

	Benign tumors	Borderline tumors	Malignant tumors	<i>p</i> -Values
CD34				
Microvessel density (vessels/mm <sup>2</sup> )	232.55±20.35	255.39±39.84	340.69±18.58	<0.001; 0.010
Percentage of microvessels in tumor (%)	4.59±0.54	8.57±0.77	8.32±0.42	<0.001; <0.001
Mean size of the vessels (μm <sup>2</sup> )	213.13±20.03	414.25±54.75	291.82±19.89	0.001; 0.044
Number of microvessels (N)	92.54±7.70	108.59±17.41	142.99±7.91	<0.001; 0.010
Endoglin (CD105)				
Microvessel density (vessels/mm <sup>2</sup> )	55.55±7.38	71.81±9.42	93.94±6.12	<0.001; 0.046
Percentage of microvessels in tumor (%)	4.84±0.42	5.06±0.82	5.41±0.25	ns
Mean size of the vessels (μm <sup>2</sup> )	1,212.25±204.51	755.26±124.10	741.65±57.53	ns
Number of vessels (N)	19.48±2.6	28.86±4.40	38.93±2.64	<0.001; 0.036

MVD: Microvessel density; ns: non-significant. Values are mean±standard error (SE).

Table IV. Clinicopathological data of the microvessels in ovarian cancer.

	Histology	Grade	Stage	Residual tumor size >1 cm	Lymph node metastases	Recurrence
CD34						
Percentage of microvessels in tumor (%)	ns	ns	ns	0.001	ns	ns
Endoglin (CD105)						
Percentage of microvessels in tumor (%)	ns	ns	0.040	ns	ns	ns
Number of vessels (n)	ns	ns	ns	0.012	ns	ns
D2-40						
Lymph vessel density (vessels/mm <sup>2</sup> )	ns	0.002	ns	ns	ns	0.013
Percentage of lymph vessels in tumor (%)	0.010	ns	ns	ns	0.008	ns
Number of lymph vessels (n)	ns	0.035	ns	ns	ns	0.042

LVD: Lymph vessel density; ns: non-significant. *p*-Values were determined by Kruskal-Wallis test.

*ovarian tumors.* There were weak but significant correlations between parameters of microvessels and the expression of VEGF-A and VEGFR3 in primary ovarian cancer. As assessed by CD34 staining, the mean size ( $r=0.3$ ,  $p=0.014$ ) and number of vessels ( $r=0.2$ ,  $p=0.049$ ) correlated positively with the cytoplasmic expression of VEGF-A.

Endoglin staining showed a weak correlation between microvessel density ( $r=0.2$ ;  $p=0.034$ ) and the number of vessels ( $r=0.2$ ,  $p=0.03$ ) with VEGFR3 expression in epithelial cancer cells.

In borderline tumors, percentage and size of vessels, as analysed by endoglin, correlated strongly with the expression of VEGF and VEGF receptors. The mean size of vessels correlated significantly with the expression of VEGF-A ( $r=0.7$ ;  $p=0.007$ ), VEGFR1 ( $r=0.6$ ;  $p=0.014$ ) and VEGFR3 ( $r=0.6$ ;  $p=0.018$ ). In addition, VEGF-D showed strong correlation with the percentage of the microvessels in the tumor ( $r=0.6$ ;  $p=0.038$ ).

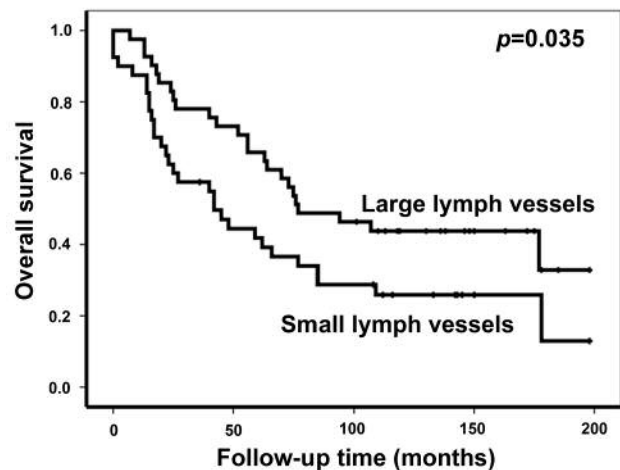


Figure 3. Kaplan-Meier curve of prognostic significance of lymph vessels. Small size of lymph vessels predicted poor overall survival in ovarian cancer.

In benign tumors, the percentage of microvessels as analysed by CD34 staining, correlated moderately negatively with the expression of VEGF-C ( $r = -0.4$ ,  $p = 0.042$ ). Furthermore, VEGFR3 correlated strongly and negatively with the percentage of the vessels ( $r = -0.5$ ,  $p = 0.002$ ) and weakly with the mean size of vessels ( $r = -0.3$ ,  $p = 0.046$ ) assessed by CD34 staining and with the number of vessels ( $r = -0.4$ ;  $p = 0.046$ ) assessed by endoglin staining. VEGFR2 did not correlate with the parameters of microvessels.

*Correlation of density and number of microvessels between CD34 and endoglin staining.* Microvessel density assessed by CD34 staining showed a weak but significant correlation with the microvessel density assessed by endoglin staining ( $r = 0.2$ ,  $p = 0.044$ ). Further, the number of microvessels correlated positively with CD34 and endoglin staining ( $r = 0.2$ ,  $p = 0.022$ ).

## Discussion

According to this study, the microvessel density was higher in malignant tumors compared to borderline and benign tumors, as assayed by endoglin and CD34 antibodies. This implies that both mature and immature vessels define malignant tumors as reported in earlier studies (13, 28).

Angiogenesis has usually been measured by investigating the most highly vascularized areas of the samples, so called hot spots, and calculating the microvessel density (13, 29). The number of hot spots has varied from one to ten in earlier studies (30, 31). Here, three hot spots were chosen as in the studies of Raspollini (2005) and Rubatt (2015) (29, 32).

High microvessel density has been linked to shorter (13) as well as longer progression free survival (14). In our study the prognostic value of microvessel density differed according to the antibody used: higher number of vessels assessed by CD34 was associated with longer progression free survival but that assayed by endoglin showed an opposite trend. According to a recent meta-analysis on microvessel density, high value of microvessel density was associated with poor prognosis and the most relevant antibody was found to be that against CD34 (13). However, we could not confirm these results.

The controversial results could be explained by the properties of the antibodies, i.e. they stain vessels in different phase of maturity. It is possible that the profuse original vessels and the new formed vessels stained by the CD34 antibody contribute to the access of the chemotherapeutics to the tumor leading to the better response and longer progression free time (33). Besides, tumors could also obtain sufficient oxygen and nutrients along the old functioning vessels for their growth and dissemination (34).

By endoglin staining the density, percentage, size and number of microvessels did not reach statistical significance

as a prognostic marker although a tendency of worse prognosis was related to the more profuse microvessels. The active neoangiogenesis of the tumor shown by endoglin staining could predict the ability of cancer cells to disseminate. On the other hand, the access of the chemotherapeutics to the tumor through immature vessels might not be as effective as through mature vessels. The different results with CD34 and endoglin staining demonstrate that they measure distinct types of vessels. Further the superiority of one or the other antibody in estimating the prognosis of ovarian cancer could not be claimed in this study contrary to earlier studies (30).

For the first time, we could demonstrate that lymphangiogenic markers in ovarian cancer were related to the more aggressive cancer and worse prognosis. The smaller size of the lymph vessels predicted significantly shorter overall survival of ovarian cancer patients. Further, higher lymph vessel density in malignant tumors predicted high grade cancer and high percentage of lymph vessels in the tumor was associated with lymph node metastases. In different types of cancer, high lymph vessel density has been linked to the lymph node metastases and small intratumoral lymph vessels have also been associated with worse prognosis (23, 34-36). However, lymph vessel parameters didn't differ between benign, borderline and malignant tumors in our study.

Interestingly, this study shows that at least at protein level, evaluated by immunohistochemistry, vessel parameters were associated differently with VEGF and VEGF receptors depending on the malignancy level of the ovarian tumor. In malignant tumors, the correlation of vessel density and number to VEGF and its receptors was weak but positive, while in borderline tumors the correlation was strong. In contrast, in benign tumors the correlation of vessels to the VEGF or their receptors was the opposite, i.e. less vessels were associated with more receptors or VEGF. This may imply differences in the regulation of angiogenesis according to the tumor type or loss of the regulatory control.

The limitations of this study include the subjective assessment of the samples, primarily in choosing the hot spots, although all samples were examined the same way. However, an image analyzing program was used and the method is widely accepted and used in various studies offering semiquantitative analysis. Another weakness of the study was its retrospective nature, and collection time of the samples, when there was not yet antiangiogenic treatment available for the patients (37).

According to these results, the features of the microvessels could be used in evaluating the prognosis and aggressiveness of epithelial ovarian cancer. Lymph vessel density, percentage and size may predict the dissemination and prognosis of ovarian cancer. By both CD34 and endoglin staining the angiogenesis was found to be more active in malignant tumors

compared to the benign ones. However, further studies are needed to examine the significance of lymph vessels in ovarian cancer patients and the great challenge would be to measure the effects of antiangiogenic therapy on the parameters of the microvessels in ovarian cancer.

## Conflicts of Interest

The Authors disclose no conflicts of interest in relation to this study.

## Authors' Contributions

MS wrote the manuscript, and analyzed statistics. HS and MA designed the study, collected the samples and clinical data, reviewed the data, and revised the manuscript. OTM analyzed the samples and statistics. SYH and VMK provided the conditions, materials, and laboratory services for the study. LKN revised the manuscript. All Authors have read and approved the manuscript.

## Acknowledgements

The Authors would like to thank Mrs Helena Kemiläinen for her skillful technical assistance. This study was supported by The Cancer Foundation of Northern Savo, The Finnish Cultural Foundation, Kuopio University Hospital Research Foundation and VTR grant.

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Received October 7, 2020

Revised October 23, 2020

Accepted October 28, 2020