# The Use of Lymph Vessel Markers to Predict Endometrial Cancer Outcome

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**Objective:** To evaluate lymphangiogenesis and lymph vessel space involvement in different subsets of endometrial cancer using podoplanin, a specific marker for lymphatic endothelium.

**Methods:** Sixty-two patients undergoing a hysterectomy with lymphadenectomy were included. Distribution of histopathologic subtypes was as follows: 30 endometrioid (48%), 18 serous (29%), 9 clear cell carcinoma (15%), and 5 carcinosarcomas (8%). Distribution of surgical stage according to the International Federation of Gynecology and Obstetrics 2009 criteria was as follows: 33 stage I (53%), 7 stage II (11%), 1 stage IIIA (2%), 15 stage IIIC1 (24%), and 6 stage IIIC2 (10%). Tumor samples were immunostained for podoplanin and the panendothelial marker, CD31. Peritumoral and intratumoral blood vessel density and lymph vessel density were assessed using an image analysis system that calculated mean vessel cross-sectional area (in micrometer squared) and vessel density (per millimeter squared). Presence of blood vessel space involvement and lymph vessel space involvement was screened for. The findings were linked with clinical outcome using Cox regression.

**Results:** Twenty-one patients (34%) experienced recurrence, and 13 (21%) died of disease. Univariate analysis showed that blood vessel space involvement was related to worse overall survival (hazard ratio, 6.59; 95% confidence interval, 1.30-120). Multivariate analyses confirmed the prognostic importance of this variable for overall survival (hazard ratio, 7.52; 95% confidence interval, 1.32–144).

**Conclusion:** Blood vessel space involvement is a prognostic marker for worse survival. Although lymph vessels were stained with the most reliable marker, podoplanin, lymph vessel density and lymphovascular space involvement do not seem to be of prognostic importance.

**Key Words:** Endometrial, Lymphatic, Podoplanin, Prognostic

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Endometrial cancer is the most common malignancy of the female genital tract in the Western world. About 75% of the cases present in early stage, and with optimal treatment, they have a 5-year overall survival (OS) of 75% to 85%.[1] Lymph node metastases are the most important prognostic marker resulting in a 5-year OS of 45%.

The genesis of lymph vessels and lymphovascular space involvement (LVSI) and the role in promoting the metastatic spread of tumor cells remain poorly understood. A relationship between patient survival and lymphatic vessel density (LVD) in different tumor types has been observed.[2] Lymphovascular space involvement has previously been investigated in endometrial cancer.[3–6] However, in the absence of specific markers for lymph vessel endothelium, distinguishing lymphatic from blood vessels can be challenging.

Podoplanin, an approximately 38-kd membrane mucoprotein originally detected on the surface of rat podocytes, is a specific marker for lymphatic endothelium and has become a valuable marker for detecting lymph vessels, for assessing LVD, and for identifying LVSI.

Clinicopathologic studies using podoplanin were performed in non–small cell lung, gastric cancer, colorectal, and oral squamous cell carcinoma.[7–11]

For this study, we evaluated LVD, blood vessel density (BVD), LVSI, and blood vessel space involvement (BVSI) in different subsets of endometrial cancer using podoplanin for lymph vessels and CD31 for blood vessels. Furthermore, we correlated the findings with clinical outcomes, including lymphatic metastasis and survival.

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## PATIENTS AND METHODS

### Patients

We selected patients who were treated for endometrial cancer at the University Hospitals Leuven between 1994 and 2007. Inclusion criteria consisted of patients with at least 1 positive node or patients with a negative complete lymphadenectomy. A complete lymphadenectomy was defined as a nodal harvest of more than 11 nodes. International Federation of Gynecology and Obstetrics (FIGO) 2009 stage[12] histological subtype and grade were determined. Patients diagnosed with stage IV disease and 6 patients who were lost during follow-up were excluded.

### Immunohistochemistry

Immunohistochemical stainings were performed on samples fixed in 10% neutral-buffered formalin and embedded in paraffin.

### Podoplanin

Paraffin sections (4 μm) were dewaxed in xylene and rehydrated with a grade of series of ethanol. Endogenous peroxidase activity was quenched by incubating slides with methanol and 30% hydrogen peroxide $H_2O_2$ for 30 minutes. After washing in Tris-buffered saline (TBS), the slides were blocked for 30 minutes. This was followed by overnight incubation at 4°C in a humidified chamber with a 1:200 dilution of antihuman podoplanin mouse antibody (Angiobio Co, Del Mar, CA). After incubation, with the primary antibody, the sections were rinsed with TBS, blocked for 30 minutes, and incubated with the secondary antibody (anti–rabbit immunoglobulins; DakoCytomation, Denmark). The biotinylated antibody was detected using streptavidin peroxidase (Roche) for 30 minutes. After rinsing in TBS, sections were treated with diaminobenzidine solution to visualize reaction products. After brief counterstaining with Mayer hematoxylin, sections were dehydrated and mounted.

### CD31

All slices were deparaffinized and put in HCl 0.2 N for 10 minutes. Slides were blocked for 30 minutes. Monoclonal mouse anti–human CD31 (DAKO, Denmark) was used as the primary antibody (dilution 1:200) in this study. Slides were incubated at 4°C overnight. Slides were then washed with a buffer and blocked for 30 minutes. Slides were sequentially incubated with polyclonal goat antimouse as the secondary antibody (DAKO, Denmark) for 30 minutes (dilution 1:50) and with mouse monoclonal APAAP (alkaline phosphatase–mouse antialkaline phosphatase) (DAKO) for another 30 minutes (dilution 1:100). Positive staining was identified by the presence of nitro blue tetrazolium. Counterstaining with methyl green was performed.

### Vessel Density and Vessel Involvement

### Lymphatic Vessel Density

Lymphatic vessel density was assessed using a KS400 image analysis system that calculated mean vessel cross-sectional area (in $\mu m^2$) and vessel density (per $mm^2$).

Two distinct methods were compared. First, the vascular hot-spot technique[13] was used. Sections were scanned at low magnification (×25, ×100) to determine the areas of podoplanin-positive vessels. Within these regions, 3 separate fields at high power (0.142-$mm^2$ field size) were examined, and the mean vessel count of the 3 fields was used. Peritumoral LVD (LVD-PT; within 1 high-power field of the tumor mass) and intratumoral LVD (LVD-IT) were recorded separately. Tumors with marked diffuse infiltrative growth without discernable tumor front were excluded in the LVD-PT analysis. Only lymphatic vessels with a distinct lumen and clusters of podoplanin-positive cells were analyzed; solitary podoplanin-positive cells were not added in the calculation.

Second, LVD was determined at random at high magnification, counting 1 in 4 fields. Peritumoral LVD was assessed moving alongside the tumor front and LVD-IT horizontally and vertically within the tumor mass. A minimum of 10 fields was counted, except in sections with not enough tumor tissue available. The mean vessel count from the 10 fields was used.

In a subset of 47 patients, we calculated equal results for LVD using the hot-spot technique and at random measurement. Therefore, only the hot-spot technique was used for the following patients.

### Blood Vessel Density

Micro–blood vessel density was assessed using only the hot-spot technique. Only CD31-positive vessels with a distinct lumen were counted. The mean count of the 3 high-power fields (0.142 $mm^2$) with the highest density was recorded. This was done intratumoral (BVD-IT) and peritumoral (BVD-PT) (vessels per millimeter squared).

### Vessel Involvement

The LVSI and BVSI were considered to be positive if at least 1 tumor cell cluster was clearly visible inside the



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podoplanin-positive (for lymph vessels) or CD31-positive (for blood vessels) vessel.

### Statistical Analysis

For survival analysis, the time of primary operation was used as the starting point for measuring the length of follow-up (in months). Univariate survival analysis for OS (using death from endometrial carcinoma as the end point or event) or progression-free survival (PFS) (using recurrence of endometrial carcinoma as the end point or event) was done using Kaplan-Meier curves and the hazard ratio (HR) based on univariate Cox regression. Non–cancer-related events were censored in this analysis. Next, a multivariate Cox regression model was constructed after multicolinearity among the variables had been investigated using variance inflation factors, correlations, and cross-tabulations. The proportional hazards assumption for Cox regression was assessed using scaled Schoenfeld residual plots, and for continuous variables, the linearity assumption was investigated using Martingale residuals. All analyses were performed using SAS v9.2 (SAS Institute, Cary, NC).



FIGURE 1. Podoplanin-positive lymph vessels with tumor invasion (original magnification ×10).

TABLE 1. Patient characteristics (n = 62)

| Variable | Statistics | Result |
|---|---|---|
| Histopathology | n (%) | |
| Endometrioid | | 30 (48) |
| Serous | | 18 (29) |
| Clear cell | | 9 (15) |
| Carcinosarcoma | | 5 (8) |
| FIGO* | n (%) | |
| I | | 33 (53) |
| II | | 7 (11) |
| IIIA | | 1 (2) |
| IIIC1 | | 15 (24) |
| IIIC2 | | 6 (10) |
| Grade | n (%) | |
| Low | | 9 (15) |
| Medium | | 7 (11) |
| High | | 46 (74) |
| LNI | n (%) | 23 (37) |
| LVSI | n (%) | 32 (52) |
| BVSI | n (%) | 41 (66) |
| LVD-PT, mm$^2$ | Median (range) | 2577 (0–10153) |
| LVD-IT, mm$^2$ | Median (range) | 5.5 (0–5527) |
| BVD-PT, microvessels/mm$^2$ | Median (range) | 210.0 (0–537) |
| BVD-IT, microvessels/mm$^2$ | Median (range) | 65.8 (0–272) |
| Recurrence | n (%) | |
| Systemic | | 14 (23) |
| Local | | 7 (11) |
| Died of disease | n (%) | 13 (21) |

*According to revised FIGO 2009 staging.[12]

## RESULTS

### Patient Characteristics

Patient (n = 62) characteristics are summarized in Table 1. A mean number of 25 lymph nodes (range, 1–69) were dissected. One hundred twenty nodes were positive on a total of 1726 that were harvested. After a median follow-up of 69 months (range, 8–158 months), recurrence was noted in 21 patients (34%), 14 of which were systemic and 7 were vaginal. The PFS event rate is 0.063 per 12 months' follow-up (mFU) or 1 event per 191 mFU, and 25% of the patients experienced recurrence within 30 months. Thirteen patients (21%) died of disease. The OS (event rate is 0.035/12 mFU or 1 event/344 mFU), and 25% of the patients died of disease within 77 months.

### Vessel Density and Vessel Involvement

For all patients, the median podoplanin-positive LVD-PT was 2577 mm$^2$ (0–10153 mm$^2$), and LVD-IT was 5.5 mm$^2$ (range, 0–5527 mm$^2$).

The median CD31-positive BVD-PT was 210.0 microvessels/mm$^2$ (range, 0–537 microvessels/mm$^2$), and 65.8 microvessels/mm$^2$ (range, 0–272 microvessels/mm$^2$) BVD-IT.

Lymphovascular space involvement (Fig. 1) and BVSI were noted in 32 (52%) and 41 patients (66%), respectively. Twenty-four patients (39%) had LVSI and BVSI.

### Relation Between LVD, LVSI, and Lymph Node Involvement

No correlation was found between LVD and lymph node involvement (LNI): the area under the ROC curve as a measure of overlap of the LVD distributions for patients with and without node involvement equaled 0.50 (95% confidence interval [CI], 0.36–0.64) for LVD-PT and 0.56 (95% CI, 0.41–0.70) for LVD-IT. Similarly, there was no relation between LVSI and LNI: 13 of 32 patients with positive LVSI have node involvement compared with 10 of 20 patients with negative LVSI (40.6% vs 50.0%; relative risk, 0.81; 95% CI, 0.45–1.54).



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### Relation Between LVD and Tumor Grade and Histology

No correlation was found between LVD and tumor grade: the area under the ROC curve equaled 0.55 (95% CI, 0.36–0.73) for LVD-PT and 0.57 (95% CI, 0.36–0.75) for LVD-IT. Nor was there a correlation between LVD and histology: 0.57 (95% CI, 0.43–0.70) for LVD-PT and 0.61 (95% CI, 0.46–0.74) for LVD-IT.

### Survival Analysis

For OS, only 13 events were recorded such that no strong conclusions can be drawn from the analysis. It also limited the number of variables that could be considered for a multivariate model. We therefore a priori selected LVD-PT, LVSI, BVSI, and LNI as the variables that will be used for a multivariate model.

For OS, univariate analysis showed that BVSI is related to worse prognosis as the hazard of the event is 6.59 times higher if BVSI is positive (HR, 6.59; 95% CI, 1.30–120; $P = 0.019$). The Kaplan-Meier curves are shown in Figure 2. In the multivariate model (Table 2), BVSI still has an independent effect on OS (HR, 7.52; 95% CI, 1.32–144; $P = 0.019$).

For PFS, we adopted the same strategy and a priori selected LVD-PT, LVSI, BVSI, and LNI for further investigation. No effects were found in both univariate and multivariate analyses (Table 2).

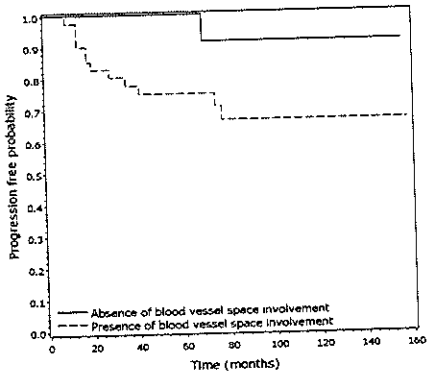



FIGURE 2. Kaplan-Meier curves for OS stratified by BVSI.

TABLE 2. Multivariate Cox regression results for OS and PFS

| Variable | HR (95% CI) | P |
|---|---|---|
| OS | | |
| LNI | 2.12 (0.61–7.23) | 0.232 |
| LVD-PT, mm | 1.053* (0.801–1.345) | 0.692 |
| LVSI | 0.90 (0.25–3.35) | 0.870 |
| BVSI | 7.52 (1.32–144) | 0.019 |
| PFS | | |
| LNI | 1.31 (0.51–3.24) | 0.565 |
| LVD-PT, mm | 1.065* (0.856–1.294) | 0.554 |
| LVSI | 1.09 (0.41–2.94) | 0.865 |
| BVSI | 1.28 (0.50–3.71) | 0.622 |

*Hazard ratio is expressed per 1000-mm increase in LVD-PT.

## DISCUSSION

Lymph node metastasis is known to be the most important prognostic factor in endometrial cancer. However, little is known of the prognostic value of the processes preceding nodal involvement, including LVD and LVSI.

Lymphangiogenic factors secreted by the tumor stimulate the formation of a peritumoral and intratumoral lymphatic network. This new drainage system may facilitate tumor growth because elimination of waste products of metabolism and tumor cell dissemination is facilitated.

These new vessels are structurally and functionally abnormal, as evidenced by increased permeability, delayed maturation, and potential for rapid proliferation (increased LVD). Because of the increased permeability, tumor cells can penetrate easier through the endothelial cell layer into lymphovascular spaces. This is regarded as an important step in the process of tumor metastases. Once LVSI has occurred, tumor cells have the potential to metastasize to the regional lymph nodes or more distantly. As a result, LVD and LVSI have been noted as an important prognostic factor in various tumors.

The standard method for assessing LVSI in endometrial cancer used to be a light microscopic examination of hematoxylin-eosin (H&E)–stained sections. Previous studies using this technique found that LVSI is an independent prognostic factor for lymph node metastases and recurrence.[1–5] Alexander-Sefre et al[6] compared the frequency of LVSI by conventional H&E staining with dual immunostaining for pancytokeratin (an epithelial marker) and CD31 (an endothelial marker) in clinically stage I endometrioid endometrial cancers. Lymphovascular space involvement was present in 54% of the cases on immunohistochemistry compared with only 16% on H&E. LYVE-1, another selective marker of the lymphatic endothelium, has been used in 2 studies in endometrial cancer. Koukourakis et al[14] revealed a higher



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LVD at the invading front of the tumor compared with myometrial areas distal to the tumor. However, no clinical correlations were made. Stefansson et al[15] concluded that a high LVD-PT was significantly associated with aggressive tumor features and decreased survival.

Based on a consensus report on immunohistochemical assessment of lymphangiogenesis in solid human tumors,[2] we used the most reliable marker, podoplanin, which has a high specificity and sensitivity.

Recently, a study has been published[16] using both podoplanin and CD31. Of 176 cases, 60% did not have any vascular invasion, 9% had only BVSI, 22% had only LVSI, and 9% had BVSI and LVSI. They observed that LVSI and BVSI (with or without LVSI) were independent prognostic markers for survival and recurrence in both univariate and multivariate analysis, although LVSI was weaker than BVSI.

Comparing these results with the current data of 62 patients, BVSI and LVSI were noted in 66% and 52%, respectively, which are higher compared with those in the study of Mannelqvist et al,[16]

According to the current data, BVSI is an independent prognostic marker only for OS, but not for recurrence. No effects were found for LVD-PT and LVSI.

Lymphatic vessel density was assessed using 2 methods: the vascular hot-spot technique and randomly. Analyzing our data, we calculated equal results using both methods, concluding that the hot-spot technique, which is a faster way of analyzing, is a reliable method to use in future research.

The strengths of this study are, first, the use of the most reliable marker for lymphatic endothelium, podoplanin; second, a selection of patients with a complete lymphadenectomy (a nodal harvest of more than 11 nodes); and third, the use of both a specific marker for lymphatic endothelium (podoplanin) and blood vessel endothelium (CD31). However, the low number of events (n = 13) is a limitation of this study; thus, no strong conclusions can be drawn from the survival analysis.

To conclude, only BVSI is a prognostic marker for worse survival. Although lymph vessels were stained with the most reliable marker, LVD-PT and LVSI do not seem to be of prognostic importance.

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