Optimizing breast cancer survival models based on conventional biomarkers and stromal parameters

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Quantitative assessment of LVSI provides important prognostic information in node-negative breast cancer patients

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Introduction

Lymph node-negative breast cancer (BC) patients are considered to have a relatively favorable prognosis, although they remain a very heterogeneous group of patients. Lymph vascular space invasion (LVSI) in both the peritumoral and the intratumoral lymph vessels has been related to various clinical end points in multiple studies [1-18]. The earliest report from the St Gallen panel that included peritumoral LVSI to recommended risk stratification was from 2005 [19], based on three published studies [5,7, 12]. Unfortunately, no uniform criteria were used in these studies. Although hematoxylin and eosin (H&E) staining is quite reliable for detection of LVSI occurring outside the contour of the primary tumor [18], use of immunohistochemical stains for D2-40 (podoplanin) and CD31 might increase LVSI detection.

Whether LVSI extent should be quantified is unclear. Eljertsen et al. [4] concluded that the addition of peritumoral LVSI to risk stratification did not identify low-risk patients who have a worse prognosis compared with other low-risk, LVSI-negative BC patients. Colleoni et al. [3] found that limited LVSI was not of prognostic significance while the presence of extensive LVSI (defined as one or more foci of LVSI in more than one tumor block) was associated with a poor prognosis. The 2007 St Gallen guidelines subsequently concluded that only extensive LVSI should be used as an adverse prognostic factor [20]. However, these guideline definitions are still relatively vague and require more evidence. Furthermore, whether this parameter provides useful information in both low-risk and high-risk patients has not been investigated. Mohammed et al. [11] showed that the extent of LVSI determined via CD34, CD31, and D2-40 staining was irrelevant for prognosis and that the presence of a single LVSI focus was sufficient to identify patients with poor outcome.

If and how LVSI should be incorporated into risk stratification of breast tumors is therefore unknown and requires further studies investigating this phenomenon and its extent in relationship to disease-free survival periods. The European Organization for the Research and Treatment of Cancer (EORTC) trial 10 854 investigated the efficacy of perioperative chemotherapy in a cohort of 2795 stage I/II BC patients [21]. In this study, we propose a number of clear morphological criteria to establish the presence of LVSI and have tried to develop a robust and simple quantitative determination for LVSI in a test cohort (N = 120) and an independent set of patients from the same trial (N = 238). The added value of this parameter in providing prognostic information in low-risk and high-risk N0 patients was also examined.

Methods

EORTC trial 10854

The perioperative chemotherapy (POP) trial (EORTC 10854) randomized 2795 patients with T1-T3, N0-2, and M0 BC to receive one course of perioperative chemotherapy or no additional treatment. 674 of these patients were reported to be premenopausal and have node-negative disease. Previous molecular studies have described 441 premenopausal node-negative patients from this trial. These 441 patients were patients that were treated at the larger centers participating in the trial and from whom tumor blocks were available. Immunohistochemial staining was carried out to assess molecular markers, as described previously [22]. Tumors were assigned to one of five intrinsic subtype categories according to the surrogate immunohistochemical definitions defined elsewhere [23].

Determination of LVSI

The presence of LVSI was determined solely on the basis of H&E slides. A sample was considered positive when there was a suspicion for LVSI that was found outside of the tumor border and met two of the following criteria: discordance between the shape of the tumor embolus and the surrounding vessel, the presence of a blood vessel in the vicinity of the suspected LVSI focus, and the presence of an endothelial lining along the suspected lymph vessel. The presence of retraction artifacts in the surrounding primary tumor was reason to not consider a lesion as LVSI. The number of LVSI foci was noted as well as the number of tumor cells in the largest tumor embolus. Tumor cells that were deemed to be necrotic were not counted. When nearby LVSI foci were within one field of ×10 magnification of each other, they were considered to be a single focus of LVSI and were not analyzed separately. All suspected LVSI were discussed by two observers (TJAD and VTHBMS). Observer disagreement was resolved by discussion, or the tumor was marked as negative for LVSI when no consensus was reached. All slides were also independently reviewed by a third observer (DVB).

Statistical analysis

All statistical analyses were carried out with SPSS (version 20.0, IBM). Relapse-free survival was defined as the time period from trial randomization to occurrence of either locoregional recurrence or distant metastases (whichever presented first, if applicable) or the last moment of follow-up. All patients for whom patient age, pathological tumor size, and tumor grade (according to Bloom-Richardson) were

available were stratified into low- and high-risk categories based on current treatment guidelines [http://www.oncoline.nl/mammacarcinoom 2013 (in Dutch)], without the inclusion of LVSI (supplementary table S1). Two systems for quantification of LVSI were tested in this patient series. The first system solely incorporated the number of affected lymph vessels in the H&E-stained sections. The second system included the number of affected lymph vessels multiplied by the number of tumor cells in the largest tumor embolus. This second parameter was termed the LVSI tumor burden (LVSI-TB). Because no evidence exists for the optimal cutoff for these parameters, this was tested by calculating sensitivity and specificity values for various cutoffs to predict relapse-free survival. These cutoff values were first determined in a test set chosen via a sequential selection of one-third of all tumors that had a LVSI score.

Subsequent disease-free survival analyses were carried out in the validation set, which incorporated the remainder of the included patients with LVSI scores. Survival curves were calculated using the Kaplan–Meier method while log-rank tests were used to assess differences in disease-free survival among different classes. For multivariate analyses investigating the independent effect of quantitative LVSI methods on hazard ratios (HRs) for disease relapse, multivariate Cox proportional hazard models were used. Factors that were associated ($P < 0.100$) with disease-free survival in univariate Cox proportional hazard models were included in multivariate analyses.

Results

Patient characteristics and follow-up

The database included a total of 441 node-negative, premenopausal patients who have been described previously [22]. Of these, 231 patients received perioperative chemotherapy (52.4%). Adjuvant chemotherapy was administered to 14 patients, who were omitted from further analyses. All available H&E-stained slides from the remaining 427 patients were collected and scored. No LVSI score was reported solely in cases when no H&E-stained slides were available or the amount of peritumoral tumor tissue was deemed too little for reliably judging LVSI status ($N = 69$). Ultimately, 358 patients were evaluated for LVSI status (supplementary figure S1, available at Annals of Oncology online). The clinico-pathological parameters for these patients and corresponding tumor tissues are listed in table 1. Disease relapse occurred in 137 patients (38.3%), and the mean disease-free survival period for these 358 patients was 8.5 years with a minimum of 1.35 and a maximum of 14.08 years.

Locoregional recurrence arose in 66 patients (18.4%), and distant metastases developed in 105 patients (29.3%).

Presence of LVSI

LVSI was detected in 81 of 358 patients (22.6%). A single focus of LVSI in the peritumoral tissue was seen in 24 cases and multiple foci of LVSI in 57 cases. When stratifying patients according to LVSI-negative and -positive cases, a statistically significant relationship was found between the presence of LVSI and disease-free survival (P = 0.002) and an increased HR for disease relapse in univariate Cox regression analyses [HR 1.743, 95% confidence interval (CI) 1.211-2.507]. When stratifying the patients for the number of LVSI, no significant difference in disease-free survival was found between patients with one single focus of LVSI and LVSI-negative tumors, regarding either mean disease-free survival or disease relapse (HR 1.423, 95% CI 0.762-2.656). Disease-free survival was statistically significantly decreased when the number of LVSI foci was equal to or exceeded 2 and seemed to decrease further with increasing number of lymph vessels affected (supplementary figure S2).

Quantitative assessment of LVSI

To determine the cutoff value that is most effective in stratifying the current group of patients regarding risk for disease relapse, several cutoff values were tested and corresponding sensitivity and specificity values determined in a test set of 120 patients from the entire cohort of 358 patients. The most optimal cutoff regarding sensitivity and specificity was found when defining a value of 60 for LVSI-TB as positive (supplementary table S2), which resulted in combined testing sensitivity and specificity superior to other cutoff values in both quantification systems. This value of 60 was verified in the validation set, where it also displayed the highest combined sensitivity and specificity.

A statistically significant relationship was found between disease-free survival and LVSI-TB for this cutoff in both the test ($P < 0.001$) and the validation sets ($P < 0.001$; supplementary figure S3). Similarly, increased HRs for disease relapse for tumors with LVSI-TB values equal to or exceeding 60 were also found in both the test (3.114, 95% CI 1.569-6.182) and the validation sets (HR 2.987, 95% CI 1.778-5.018) in univariate analyses. Disease-free survival and HRs for disease relapse did not differ between tumors that were LVSI-TB low ($0 < \text{LVSI-TB} < 60$) and tumors for which no LVSI was found in both datasets (HR 0.709, 95% CI 0.217-2.318 for the test set; HR 0.906, 95% CI 0.450-1.821 in the validation set). Therefore, these latter two categories

were considered together as LVSI-TB low. This parameter was investigated in multivariate analyses in the validation set. All parameters that were associated with disease-free survival in univariate analyses in the group of 238 patients were investigated in multivariate analyses (supplementary table S3). Parameters included in the multivariate model were patient age (< 40 or ≥ 40 years), intrinsic subtypes, tumor grade (I or II/III), perioperative chemotherapy (yes or no), and LVSI-TB (< 60 or ≥ 60). LVSI-TB was independently associated with an unfavorable prognosis, independent of other prognostic parameters (HR 2.366, 95% CI 1.369-4.090).

Addition to low- and high-risk patients

We then evaluated the prognostic influence of LVSI-TB in both low- and high-risk N0 patients in the validation set. In the low-risk N0 group, a significantly reduced disease-free survival period was seen when high LVSI-TB was detected ($P < 0.001$; figure 1A). The prognosis for this group of patients with extensive LVSI-TB did not differ from that of high-risk patients. In the high-risk patients, LVSI-TB was again associated with a decreased disease-free survival ($P = 0.007$; figure 1B, supplementary table S4).

Discussion

We propose a strict definition for LVSI detection in H&E-stained slides and method for quantification of LVSI in a series of breast tumors treated as part of the POP trial. This quantitative method was based on the multiplication of the number of lymph vessels involved by the number of cells in the largest tumor embolus. Although LVSI has been associated with clinical outcome in many studies, the majority of these did not describe clear histological criteria or apply any form of LVSI quantification. A dichotomous classification of LVSI (present versus absent) groups all tumors with LVSI into one group even though the extent to which lymph vessels are affected varies considerably. This might lead to overtreatment because the presence of small tumor emboli might not be enough evidence to upgrade otherwise low-risk patients to a high-risk category. The number of tumor cells that have metastasized to regional lymph vessels is likely to indicate the chance of developing metastases. This assumption is supported by the results of this study, where we found that the amount of LVSI tumor cells had a great impact on patient survival. When this LVSI-TB was low (< 60), we identified no statistically increased HR for disease relapse; the HR did increase significantly for patients when this value equaled or exceeded 60.

Colleoni et al. [3] were first to quantify LVSI in a series of 2606 patients and concluded that only patients with extensive LVSI had a statistically significant increased HR for

disease relapse. The study did not investigate further whether adjustments to this cutoff could be made, leaving open the question of whether this approach is an optimal risk stratification for LVSI. By providing a numerical estimation of the LVSI-TB, we have attempted to define LVSI cutoff values with an optimal sensitivity and specificity. We therefore feel that our study gives strong evidence that quantification of LVSI offers valuable information and supports the implementation of this parameter in the standard pathological documentation.

In contrast to our findings and those published by Colleoni et al. was the study of Mohammed et al. [11] which did not reveal a relationship between LVSI extent and disease-free survival. Although they reported a statistically significant relationship between LVSI and disease-free survival on multivariate analysis, no association was found between greater extent of LVSI and clinical outcome. This might be because Mohammed et al. used immunohistochemistry to aid LVSI detection compared with H&E-stained sections used in our study. Despite this difference in methodology, the percentage of detected LVSI foci in our study, Mohammed et al. and Colleoni et al. was comparable.

We suggest that the strict criteria we used ensure a high reliability for determining LVSI (supplementary figure S4A). More studies should be done to establish interobserver variability of LVSI using those criteria. The results of our study support the notion that H&E slides can be used as an important primary tool for LVSI detection and can directly provide this prognostic information. In cases of doubt (supplementary figure S4B and C), the presence of LVSI can be confirmed with D2-40 immunohistochemistry staining, which unfortunately was not available for this study.

A drawback of our study was the limited number of patients enrolled compared with other studies that have investigated LVSI. However, strong points were the relatively long period of follow-up and the relatively homogeneous group of included patients. These patients were all derived from one larger clinical trial in which the treatment was relatively similar and that used randomized intervention. The POP trial investigated the efficacy a short-course of chemotherapy. This is not comparable to modern-day chemotherapy regimens. The impact of chemotherapy on disease-free survival remains an important question and should be assessed in subsequent studies. Application of this quantitative LVSI parameter for node-negative BC patients is particularly appealing because it requires no extra costs and can be directly applied on the same H&E-stained slides that are used in the BC diagnosis.

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Table 1. Characteristics of the included patients.

		Test (*N* = 120)	Validation (*N* = 238)
Mean age (range)	44 (24–63)	45 (28–60)	44 (24–63)
pT1	167	54	113
pT2	152	52	100
pT3	1	1	0
Grade I	121	39	82
Grade II	113	33	80
Grade III	114	38	76
Luminal A	111	47	64
Luminal B (HER2 negative)	164	43	121
Luminal B (HER2 positive)	41	20	21
HER2 positive (non-luminal)	5	0	5
Basal-like	28	6	22
Low-risk	108	36	72
High-risk	230	77	153
p53 low	291	102	189
p53 high	66	18	48
MVD low	79	23	56
MVD high	203	76	127
Relapse negative	221	74	147
Relapse positive	137	46	91
Lumpectomy	297	102	195
Mastectomy	61	18	43
No tamoxifen	346	117	229
Adjuvant tamoxifen	9	2	7
No perioperative chemotherapy	175	56	119
Perioperative chemotherapy	183	64	119

Table S1. Definitions of low- and high-risk N0 breast cancer patients.

High risk N0	Patient age < 35 years (unless pT-size < 10 mm and tumor grade = 1)
	Patient age ≥ 35 years and pT-size > 10 mm and ≤ 20 mm and tumor grade = 2 or 3
	Patient age ≥ 35 years and pT-size > 20 mm and any tumor grade
Low risk N0	Patient age < 35 years, pT-size < 10 mm and tumor grade = 1
	Patient age ≥ 35 years and pT-size ≤ 10 mm and any tumor grade
	Patient age ≥ 35 years and pT-size ≥ 10 mm and ≤ 20 mm and tumor grade = 1

Table S2. Determination of the cut-off value for quantitative LVSI determination in test set.

LVSI foci cut-off	Sensitivity (%), specificity (%)	LVSI tumor burden	Sensitivity (%), specificity (%)
1	30.4%, 83.8%	**20**	23.9%, 83.8%
2	19.6%, 89.1%	**40**	23.9%, 89.2%
3	13.0%, 94.6%	**60**	23.9%, 94.6%
4	6.5%, 98.7%	**80**	15.2%, 94,6%
		100	10.9%, 95.9%
120		6.5%, 98.6%	
140		4.3%, 98.6%	

Table S3. Univariate and multivariate Cox regression analyses predicting disease relapse in the validation set.

Parameter	Univariate			Multivariate		
	Hazard ratio	95% CI	*P*-value	Hazard ratio	95% CI	*P*-value
Age < 40	1.913	1.251-2.927	0.003	1.776	1.150-2.741	0.010
Age ≥ 40	1.000	Ref		1.000	Ref	
pT1	1.000	Ref				
pT2	1.124	0.729-1.733	0.597			
Luminal A	1.000	Ref		1.000	Ref	
Luminal B (HER2 negative)	1.963	1.134-3.399	0.016	1.671	0.881-3.170	0.116
Luminal B (HER2 positive)	2.501	1.141-5.483	0.022	1.711	0.683-4.287	0.252
HER2 overexpression	3.206	0.937-10.966	0.063	2.108	0.538-8.260	0.285
Basal-like	1.684	0.727-3.903	0.224	1.219	0.455-3.266	0.693
Grade I	1.000	Ref		1.000	Ref	
Grade II	1.145	0.665-1.973	0.626	0.879	0.483-1.599	0.672
Grade III	2.050	1.240-3.389	0.005	1.302	0.688-2.464	0.417
MVD low	1.000	Ref				
MVD high	0.966	0.593-1.571	0.888			
p53 low	1.000	Ref				
p53 high	1.156	0.704-1.900	0.567			
Lumpectomy	1.253	0.748-2.099	0.392			
Mastectomy	1.000	Ref				
No tamoxifen	1.000	Ref	0.769			
Tamoxifen	1.118	0.376-3.759				
No perioperative chemotherapy	1.000	Ref		1.000	Ref	0.324
Perioperative chemotherapy	0.702	0.464-1.062	0.094	0.833	0.520-1.242	
LVSI tumor burden low (< 60)	1.000	Ref	< 0.001	1.000	Ref	0.002
LVSI tumor burden high (> 60)	2.987	1.778-5.018		2.366	1.369-4.090	

Table S4. Analysis in low- and high-risk N0 patients from the validation set.

Parameter	Low-risk N0 patients	High-risk N0 patients
N	72	153
LVSI-TB low (relapse)	67 (17)	134 (50)
LVSI-TB high (relapse)	5 (4)	19 (13)
Hazard ratio (95% CI)	8.362 (2.715-25.758)	2.276 (1.235-4.197)
P-value	< 0.001	0.007

Figure 1. Relapse-free survival in 238 patients stratified for the LVSI tumor burden in two categories (1: No LVSI or LVSI-TB < 60, 2: LVSI ≥ 60) in low- (A) and high-risk (B) patients.

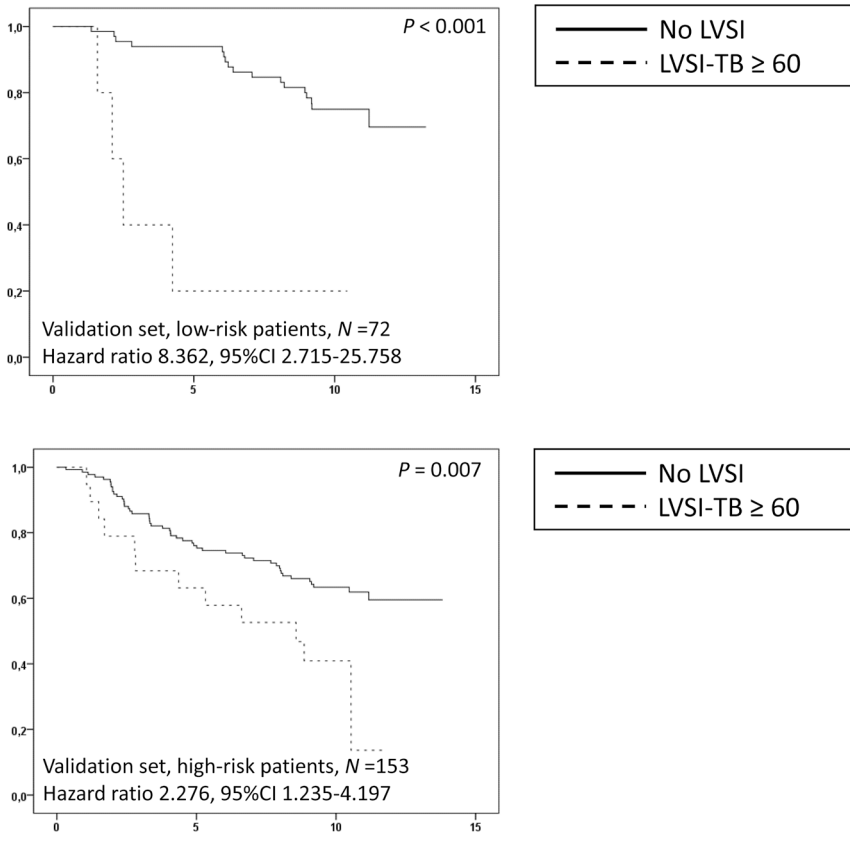

Figure S1. Flow chart of the patients included in the EORTC trial 10854 (POP), previous sub-analysis and the current study. (A) This selection was made as this group of patients had a relatively favourable response to perioperative chemotherapy and were thus further studied. (B) Determination of low- or high-risk status was not possible for 13 patients due to missing data concerning patient age, pathological tumor size and/or Bloom-Richardson grade.

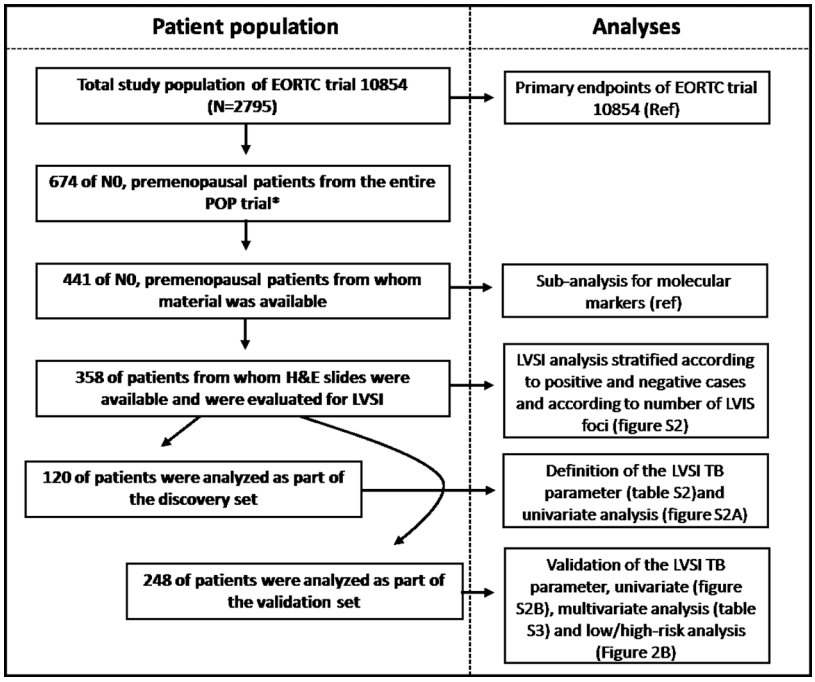

Figure S2. Relapse-free survival in 358 patients stratified for the number of LVSI foci.

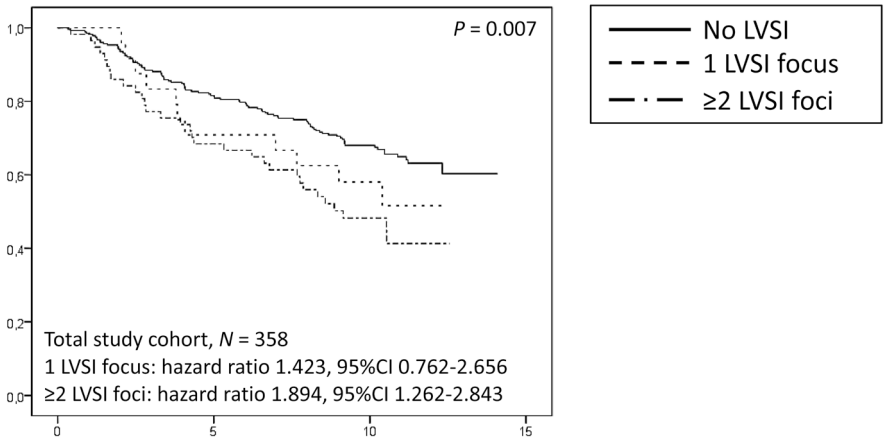

Figure S3. Relapse-free survival in the training and validation set of patients stratified for the LVSI tumor burden stratified according to three categories (1- no LVSI, 2- LVSI-TB < 60, 3- LVSI-TB ≥ 60).

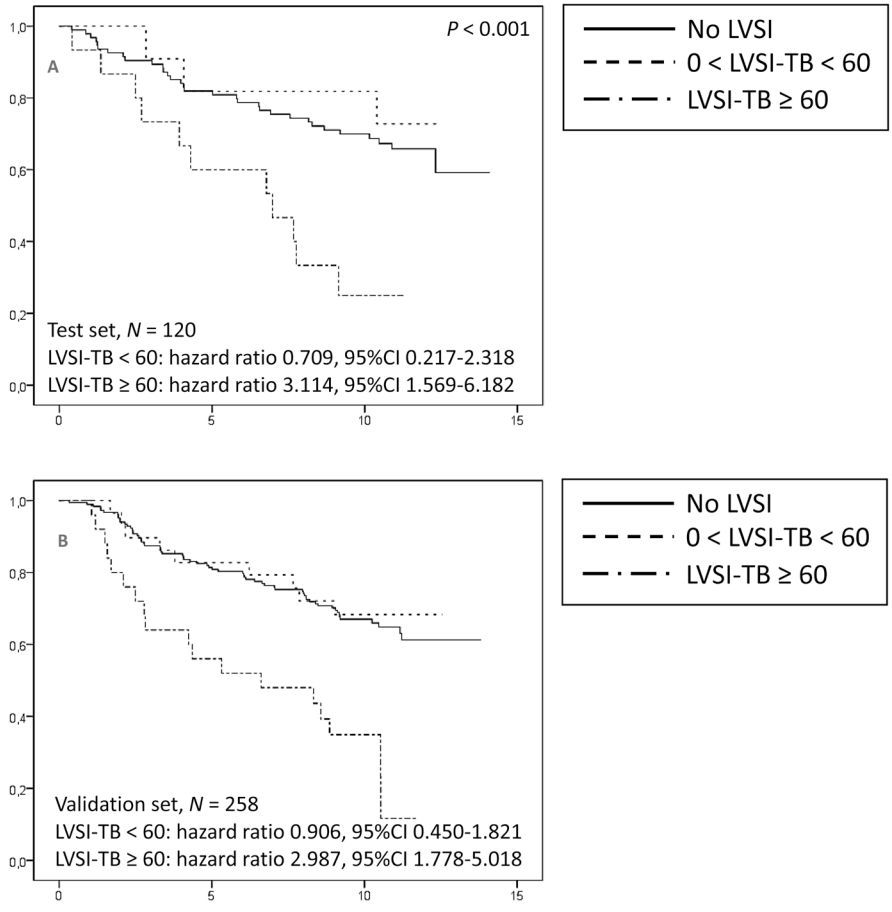

Figure S4. Three suspected cases of LVSI. The top figure represents a lesions that adheres to all scoring criteria. The middle figure shows a lesion that is suggestive for an LVSI focus, but lacks a discernible space between the tumor embolus and the lymph vessel. The bottom figure also displays lesions that resemble LVSI, but due to the retraction artefacts in its immediate surroundings, these cannot be scored as such.