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Prognostic relevance of angiogenesis in stage III NSCLC receiving multimodality treatment

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Short title: Prognosis of microvessel density in stage III NSCLC

Keywords: Angiogenesis, lymph node metastases, microvessel density, multimodality treatment, non-small cell lung cancer

Abstract

Compelling evidence indicates that microvessel density (MVD) is a prognostic marker in early non-small cell lung cancer (NSCLC). However, its role in lymph node metastases (LN) in stage III NSCLC receiving multimodality treatment is unknown.

LN of 142 patients with stage III NSCLC treated in a trial of the German Lung Cancer Cooperative group, were evaluated for MVD. Median follow-up was 7.39 years. MVD was correlated with demographic and tumor-related variables and survival.

MVD (median 33.9) did not correlate with survival. Though, in multimodality treated stage IIIA patients receiving tumor resection with negative margins (R0), those with a high MVD had significantly prolonged overall survival with a median of 4.96 years compared to 1.99 years for those with low MVD (p=0.041). Cox regression analysis revealed that MVD was a prognostic factor in R0-resected stage IIIA (hazard ratio 0.417). Furthermore, a significant correlation of MVD to stage was observed with significant lower MVD in stage IIIA than IIIB (p=0.0062) and to histological subtype with adenocarcinoma revealing the highest scores (p=0.0001).

Increased angiogenesis within LN is a prognostic indicator for better survival in NSCLC patients. Thus, measurement of MVD might be useful in selecting patients for future neoadjuvant treatment decisions.

Introduction

Angiogenesis - the formation of new microvessels from the pre-existing vasculature in a complex, multistep process - is involved in the growth, maintenance and metastasis of most solid tumors [1]. Several reports have demonstrated a significant correlation between neovascularization assessed by intratumoral microvessel density (MVD) with clinicopathological factors and patients' outcome in a variety of tumors [2].

Regarding the prognostic relevance of angiogenic activity in non-small cell lung cancer (NSCLC) as expressed by the intratumoral MVD, high MVD has been identified as a prognostic factor predictive of metastasis and poor survival [3]. Furthermore, high expression of vascular endothelial growth factor (VEGF) and other angiogenic growth factors within the primary tumor are considered as independent indicators of diminished outcome in NSCLC patients [4]. However, most of the studies have been conducted on early stage lung cancer and little is known about the prognostic impact of angiogenesis in metastases - especially lymph node metastases - of NSCLC patients.

In locally advanced stages IIIA and IIIB of NSCLC with mediastinal lymph node metastases, locoregional treatment alone with surgery or radiotherapy results in disappointing long-term outcome. Multimodality treatment approaches with preoperative chemo(radio)therapy followed by surgery could improve outcome and enable surgery, even in stage IIIB disease [5, 6]. However, these treatment options are still under development [7]. The increasing availability of antibody therapy and tyrosine kinase inhibitors rise interest to identify patient subgroups that will benefit from additional systemic treatment, even in the multimodality approach. The inhibition of tumor angiogenesis has already been evaluated as a therapeutic strategy with great promise for the development of new lung cancer therapies. A wide range

of different anti-angiogenic drugs are currently under investigation or have already been licensed for the treatment of lung cancer [4].

In the present study we examined the clinical significance of neovascularization in lymph node metastases in patients with locally advanced NSCLC before the initiation of a multimodal treatment strategy within a trial of the German Lung Cancer Cooperative group [8] by correlating MVD with survival, response to therapy and further prognostic factors.

Materials and Methods

Patients and treatment

Paraffin-embedded specimens of mediastinal lymph node biopsies from 142 patients with locally advanced, untreated stage III NSCLC were studied. Criteria for including patients to the present investigation were sufficient representative pretherapeutical biopsy material for MVD-analysis and treatment within a randomized phase III trial of the German Lung Cancer Cooperative Group (GLCCG) that was approved by the institutional review board and registered at www.clinicaltrials.gov (NCT00176137) [8]. Briefly, in this protocol, patients with stage III NSCLC (invasive mediastinal staging obligatory) were stratified according to centre and stage (IIIA or IIIB) and then randomized to therapy. Therapy consisted in the experimental arm of 3 cycles of cisplatin 55 mg/m^2 (d 1+4) / etoposide 100 mg/m^2 (d 1-4) (PE), followed by hyper-fractioned radiotherapy (hfRT) (45 Gray; 2 x 1.5 Gray/d) with concurrent carboplatin 100 mg/m^2 / vindesine 3 mg (d 1, 8, 15), then surgery. In inoperable patients or with R1/2-resection additional hfRT (24 Gray; 2 x 1.5 Gray/d) was offered. In the control arm patients were scheduled to receive 3 cycles PE, followed by surgery and then conventionally fractionated radiotherapy (1.8 Gray/d) with 54 Gray or, if patients were inoperable or received R1/2-resection, with 68.4 Gray. Patients were excluded if written consent to the protocol or sufficient histological specimens were not available.

The following variables were evaluated for their distribution in the patient cohort and for possible correlations with outcome: patient age, gender, histological subtype, randomization to treatment arm, stage, lymph node status at the time of diagnosis, response to therapy, resection margins of the primary tumor (negative [R0] versus positive [R1, R2]) and complete or incomplete resection. Complete resection was defined as negative resection

margins (R0) without metastastic involvement of the uppermost removed mediastinal lymph node.

Immunhistochemical studies and evaluation of microvessel density

Serial sections of paraffin embedded biopsy specimens were processed for immunohistochemical identification of microvascular endothelial cells with an anti-CD31 antibody (clone JC/70A, Dako) (working dilution 1:100). Immunhistochemical localization was performed by the alkaline phosphatase/anti-alkaline phosphatase double bridge technique (Dako-APAAP kit, Dako). Before staining, tissue sections were deparaffinized in xylene, rehydrated in a graded ethanol series, and treated with Proteinase K for antigen retrieval. The primary antibody was applied overnight at 4^0 C. Subsequent steps were performed according to the manufacturer's instructions. The fast red substrate (Dako) was employed for revelation of phosphatase activity (10 minutes at room temperature). Sections were counterstained with 0.1 % (w/v) hematoxylin.

The degree of angiogenesis was determined by the count of microvessels within 3 independent hot spots per section and in 2 sections as described before [9, 10]. MVD was defined as the mean count of microvessels per 0.26 mm^2 field area (i.e. x400 field). The median interobserver and intersectional variability was low (2% and 4%, respectively). Median MVD of the entire group was predetermined to classify patients in two groups with high (> median) and low (≤ median) MVD according to an international consensus report [10].

Statistical analysis

The association of (classified) MVD and categorical prognostic factors (i.e. gender, histological subtype, randomization to arm A or B, stage, lymph node status, resection

margins and resection status) were examined using Fisher's exact test. In order to examine the association of (classified) MVD and patient age, the U-test was applied.

The distributions of the time-to-event variables were estimated using the Kaplan-Meier method, and comparisons were based on the log-rank test. Median follow-up was estimated by means of the inverse Kaplan-Meier method. All potential prognostic factors assessed and MVD were tested using the Cox proportional hazards model.

All p-values reported are two-sided with a significance level of 0.05. Statistical analyses were intended to be explorative and not confirmative. No adjustment for multiple testing was carried out. All calculations were performed using the SAS package (SAS Institute Inc., Cary, NC, USA).

Results

Patient characteristics

Subjects of the present study were 142 patients with NSCLC treated within a phase III trial of the GLCCG [8]. There were 114 males and 28 females with a median age of 59 years (range 33-69 years). Patient characteristics are shown in table 1. 65 patients had squamous cell carcinoma, 62 adenocarcinoma and 15 patients large cell carcinoma. 77 patients were randomized to the experimental and 65 to the control arm. 59 patients were diagnosed with stage III A, 83 with stage III B. Surgery was performed on 88 of all patients with 73 patients receiving negative resection margins (R0) and 51 patients with a complete resection. 38 of the patients with R0 resection had stage IIIA and 35 stage IIIB disease.

Microvessel density in non-small cell lung cancer

Median microvessel count for all patients was 33.9 (x400 field, 0.26 mm^2) with an interquartile range from 9.25 to 69.5. Patients were classified in two groups with high (> 33.9) and low (≤ 33.9) MVD, based on the median MVD of the entire group. Representative lymph node biopsy tissue specimen from lung cancer patients are shown in figure 1a for a tumor with low and in figure 1b for a tumor with high MVD.

A significant correlation of MVD with stage was noticed (p=0.0062). In stage III A median MVD was 22.4 while in stage III B median MVD was 40.6. Concerning the nodal status, a high MVD correlated significantly with an advanced lymph node status (N3) (p=0.019). Furthermore, a statistical significant association could be detected between MVD and the histologic subtype (p=0.0001). While 69.4% of patients with adenocarcinoma had a high MVD, it were only 33.2% in squamous cell carcinoma. Moreover, a statistical significant association between MVD and gender could be detected (p=0.019). There was no statistically

significant correlation between MVD and patient age, resection margins, resection status or treatment arm (data not shown).

Correlation of microvessel density with overall and relapse free survival

The median follow-up period was 7.39 years (range 0.14 – 9.63 years). For all 142 patients analyzed, overall survival (OS) and progression free survival (PFS) did not differ between patients with high versus low MVD (p=0.80 and p=0.87, respectively) (figure 2a and 2b). However, in stage IIIA patients receiving tumor resection with negative margins (R0) OS was significantly prolonged in those with a high MVD with a median of 4.96 years compared to those with a low MVD with a median survival of 1.99 years (p = 0.041). Also, PFS was prolonged (2.57 versus 1.62 years) without reaching statistical significance (p=0.089) (figure 3a and 3b). To rule out that intercurrent disease might have interfered with the OS analysis a mortatlity-analysis was performed which showed that most deaths were tumor related and did not alter the results of this analysis (data not shown).

The entire group of patients underwent further subgroup analysis according to lymph node status, treatment arm, resection margins and resection status. These factors were selected as they might impact outcome and interfere with the predictive impact of MVD [6, 8]. Subgroup analyses were performed upon the median MVD of the entire patient population (33.9) and upon median MVD of the respective subgroups.

In the analysis of stage IIIA, the subgroup median MVD was 22.4. Here, with a median follow up of 9.31 years, PFS was significantly prolonged in patients with a high MVD (median PFS 1.84 years) compared to a low MVD (median PFS 0.89 years), (p = 0.05) and for those IIIA patients with negative resection margins (p=0.03). OS showed a similar association in the stage III subgroup (2.26 versus 1.62 years), however, without statistical significance (p=0.15).

For patients with stage III B, N2 or N3-lymph node status, treatment in the experimental or control arm, negative resection margins or complete resection, OS and PFS did not differ regarding high versus low MVD (data not shown).

Univariate Cox regression analysis identified MVD in patients with stage IIIA and negative resection margins as an independent prognostic survival factor (hazard ratio 0.417, p=0.04) and in the subgroup analysis for stage IIIA (cutoff 22.4) as a prognostic parameter for PFS (hazard ratio 0.580, p=0.05). Furthermore, multivariate Cox regression analysis revealed that resection status (complete versus incomplete) was an independent prognostic factor for survival in the overall patient population (p=0.009).

Discussion

Aim of the present study was to examine the clinical significance of neovascularization in lymph node metastases of patients with stage III NSCLC prior to the initiation of polychemotherapy within the context of a multimodality treatment strategy. Despite progress in understanding the molecular biology of NSCLC its treatment remains challenging. Until today there is a lack of clinical stratifying categories before initiating chemotherapy in locally advanced NSCLC to predict the response to therapy. Current investigations have revealed that angiogenesis plays a significant role in the pathogenesis of NSCLC and in the mechanisms of disease progression [4].

The present investigation demonstrates for the first time in a large group of patients a significant association of MVD within mediastinal lymph node metastases with survival of stage IIIA NSCLC patients who received multimodality treatment including tumor resection with negative margins. Patients with a high degree of MVD within the metastases had a significant enhanced survival than patients with low microvessel density.

The association of MVD and prognosis in NSCLC has already been reported by several others. Our data are in line with a report by Chandrachud et al. who showed that survival time was generally longer for NSCLC patients with higher vascularity [11]. However, in contrast to our and Chandrachuds results, most other authors describe that high MVD is a poor prognostic factor for survival in NSCLC. Yet, several factors have to be considered:

First, most of these reports investigated MVD within primary tumors while we aimed to determine MVD within lymph node metastases. A study by Angeletti et al. investigating a comparable study population as ours came to contrary results, showing that a high MVD within the primary tumor was a negative prognostic factor [12]. This might assume that comparing MVD within a primary tumor and lymph node metastases is not reliable. Support

of this hypothesis comes from a study by Edel et al. who demonstrated only varying associations between the level of angiogenesis in individual breast cancer tumors and their metastastic lymph nodes [13]. Furthermore, Guidi et al. showed that only MVD within lymph node metastases, but not within primary mammary carcinomas correlated to survival [14].

Moreover, most reports on the role of MVD in NSCLC determined angiogenesis in early stage lung cancer while we explored patients with locally advanced NSCLC. As prognosis differs remarkably within different stages of NSCLC, comparisons have to be made with caution.

However, the presumably most important difference might be that other authors only investigated surgically treated patients while our patients were treated within a multimodality therapy concept. The integration of intensive polychemotherapy into a multimodality treatment strategy has led to distinct prognostic improvements in stage III NSCLC [5], which might be an explanation for better survival of patients with a high degree of microvessel density in our report. As tumor microcirculation is an important factor in drug delivery to cancer cells [15], hypothetically a higher density of blood vessels could improve accessibility of the chemotherapy to proliferating lung carcinoma cells, i.e. the efficacy of drug delivery might be much higher in a tumor with a high degree of microvessels than in a tumor with a low MVD. Support of this hypothesis comes from a study by Volm et al. who showed that MVD was significantly reduced in chemotherapy resistant NSCLC tumors when compared with chemotherapy sensitive tumors [16]. Moreover, direct anti-angiogenic mechanisms of cytotoxic chemotherapy by induction of endothelial cell apoptosis may contribute to the eradication of tumor cells. Indeed, direct toxic effects on endothelial cells as well as real anti-angiogenic effects have been described in in vitro and in vivo models for different cytostatic agents (e.g. anthracyclins, vinca alkaloids, paclitaxel) [17-20]. This hypothesis might also explain the difference of the results by Angeletti et al. and ours, most probably related to

different treatment modalities. In our study all patients received intensive polychemotherapy while only about a third of the patients in the report by Angeletti et al. were treated chemotherapeutically [12]. Furthermore, the effect of radiation or chemoradiotherapy has to be considered as hypoxia within a tumor causes resistance to radiotherapy. Hypothetically, oxygenation might be improved within a highly vascularized tumor lesion leading to enhanced response to therapy and thereby survival. This hypothesis is supported by several reports which have already shown that higher MVD significantly correlates with better response and survival in solid tumors treated with chemo-radiotherapy [15, 21]. Finally, our observation that MVD correlated to better survival in multimodality treated stage IIIA patients with negative resection margins (R0) but not in those with a complete resection is remarkable. A complete resection is defined as a resection with negative without metastastic involvement of the uppermost removed mediastinal lymph node and is an indicator for enhanced survival in stage III NSCLC undergoing multimodality treatment [8]. Our finding might therefore give support of our assumption that lymph node metastases with an increased vascularization have an improved response to chemo/radiotherapy than those with low MVD. Even if the uppermost mediastinal lymph node after R0 resection was involved, patients with a high MVD had an enhanced survival than those with a low MVD within the LN metastasis which might be attributed to the effects of chemo-radiotherapy.

Moreover, some of the discrepancies to the afore mentioned reports may also be explained by the fact that details of the methodology used to assess MVD can influence its value as a prognostic indicator, e.g. the antibody (CD31, CD34, von Willebrand factor) or whether MVD is assessed at the periphery or the center of the tumor [22]. According to an international consensus, CD31 is the proposed standard for the quantification of intratumoral MVD [10]. However, it has to be discussed critically that estimation of MVD within lymph node metastases by CD31 might result in false positive results [23]. Nonetheless, our

estimations of MVD within the lymph nodes were only performed in large metastatic lesions replacing the original lymph node structure.

Yet, it has to be discussed critically why MVD only was a positive predictor of survival in stage IIIA. However, one has to consider that stage III NSCLC is a heterogeneous collective of patients and that survival in stage IIIB NSCLC is significantly diminished compared to stage IIIA. Furthermore, we had to observe that R0 resection in stage III B patients could only be performed in a lower percentage than in stage III A, suggesting that the unfavorable resection status in stage IIIB patients influenced outcome the most. This hypothesis was supported by the finding that resection status was an independent prognostic factor for survival in the overall patient population analyzed. Furthermore, survival in locally advanced NSCLC depends on further aspects such as comorbidity and other molecular and immunological factors. Still, we cannot rule out that our subgroup analysis resulted in a large increase in the Type I error. However, our analyses are not of confirmatory level of evidence and we did not evaluate a pre-specified null hypothesis as our analyses were explorative. Furthermore, in a large subset of our analyses we found a trend towards a better survival in patients with high MVD, however without reaching statistical significance.

Adenocarcinoma is the most common type of lung cancer in most recently reported series, and is the most frequent histologic type in women and non-smokers of either sex [24]. In regard to this, our observation that MVD correlated significantly to the histological subtype and that adenocarcinoma significantly more frequently had a high MVD is remarkable. Similar results were obtained by Yuan et al. demonstrating significant higher MVD for patients with adenocarcinoma than for squamous cell carcinoma [25].

A further notable result of our investigation was the correlation of MVD to stage and to lymph node status. These observations are in line with others [25-27] underlining the

important impact of angiogenesis in the progression of NSCLC and giving proof of the reliability of our findings.

In conclusion, the present study provides evidence for the prognostic significance of MVD within lymph node metastases of stage III A NSCLC patients receiving resection with negative margins in a multimodality approach. Higher survival rates were demonstrated for patients with increased MVD. Thus, MVD might be a potentially useful predictive marker in patients treated within a multimodal treatment strategy and should be further explored as a potential tool for treatment stratification.

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Conflict of interest statement

Nothing to disclose.

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Table 1: Patient characteristics

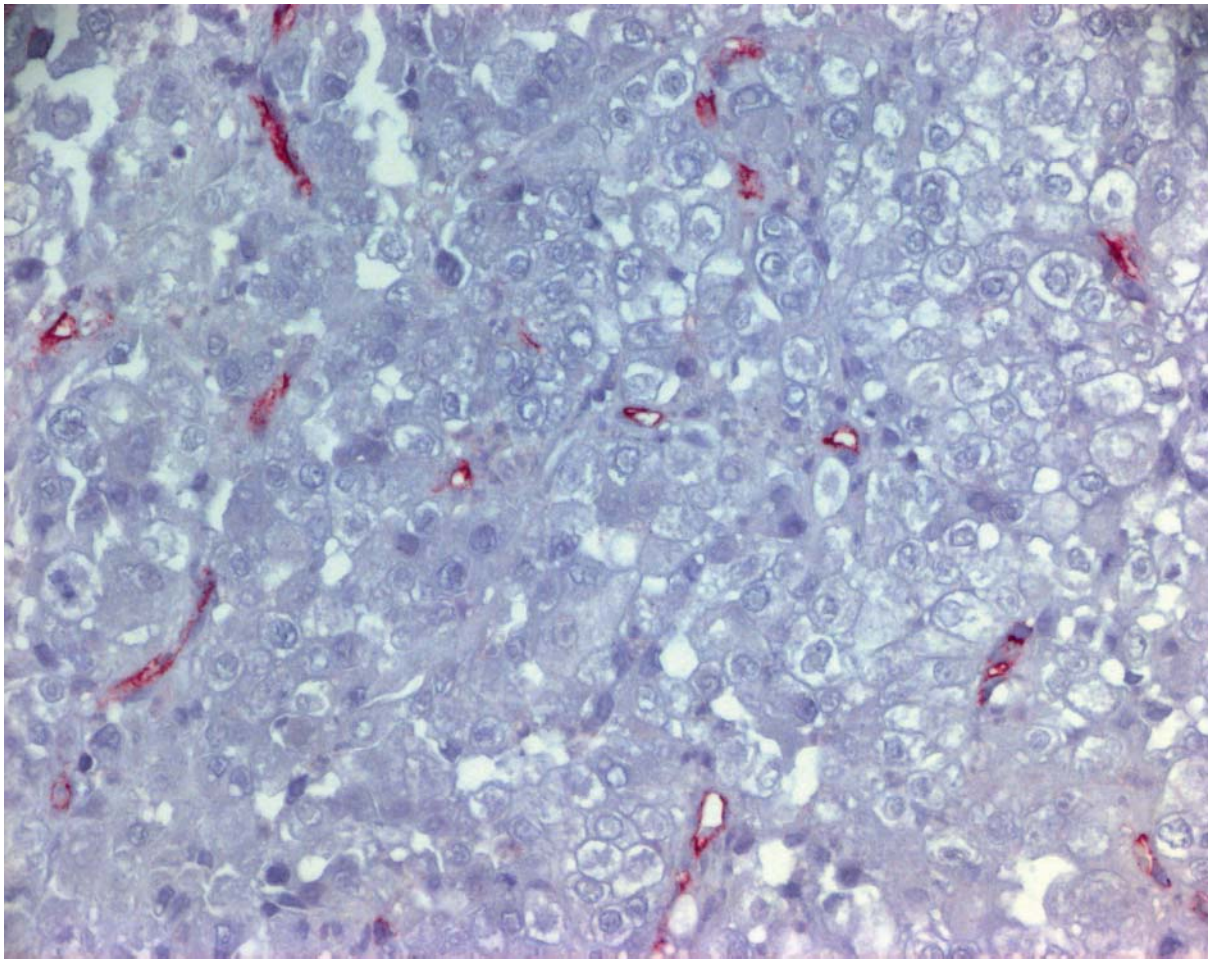
Feature	**Number *** (n=142)	**High MVD (n=71)**	**Low MVD (n=71)**	**p-value**
Median age (range)	59 (33-69) years	59 years (33-69)	59 years (37-69)	p=0.5468
Gender				
Male	114	51	63	p=0.0192
Female	28	20	8	
Histological subtype of NSCLC	65	21	44	p=0.0001
Squamous cell carcinoma	62	43	19	
Adenocarcinoma large cell carcinoma	15	7	8	
Stage				
III A	59	21	38	p=0.0062
III B	83	50	33	
Randomization to				
Experimental arm	77	39	38	p=1.0000
Control arm	65	32	33	
Surgery performed	88	40	48	
Tumor resection margins				
negative	73	34	39	p=0.5734
positive	5	1	4	
Exploratory thoracotomy	10	5	5	
Tumor resection (in negative resection margins)				
Complete	51	27	24	p=0.1273
Incomplete	22	7	15	

MVD: microvessel density; high > median; low ≤ median

* if not otherwise stated

Legends

Figure 1: Immunohistochemical staining of NSCLC lymph node metastases tissue slides with anti-CD31 antibodies for the quantification of microvessel density. (1a) Section from a specimen with low microvessel density. (1b) Section from a specimen with high microvessel density. Original magnification x200.



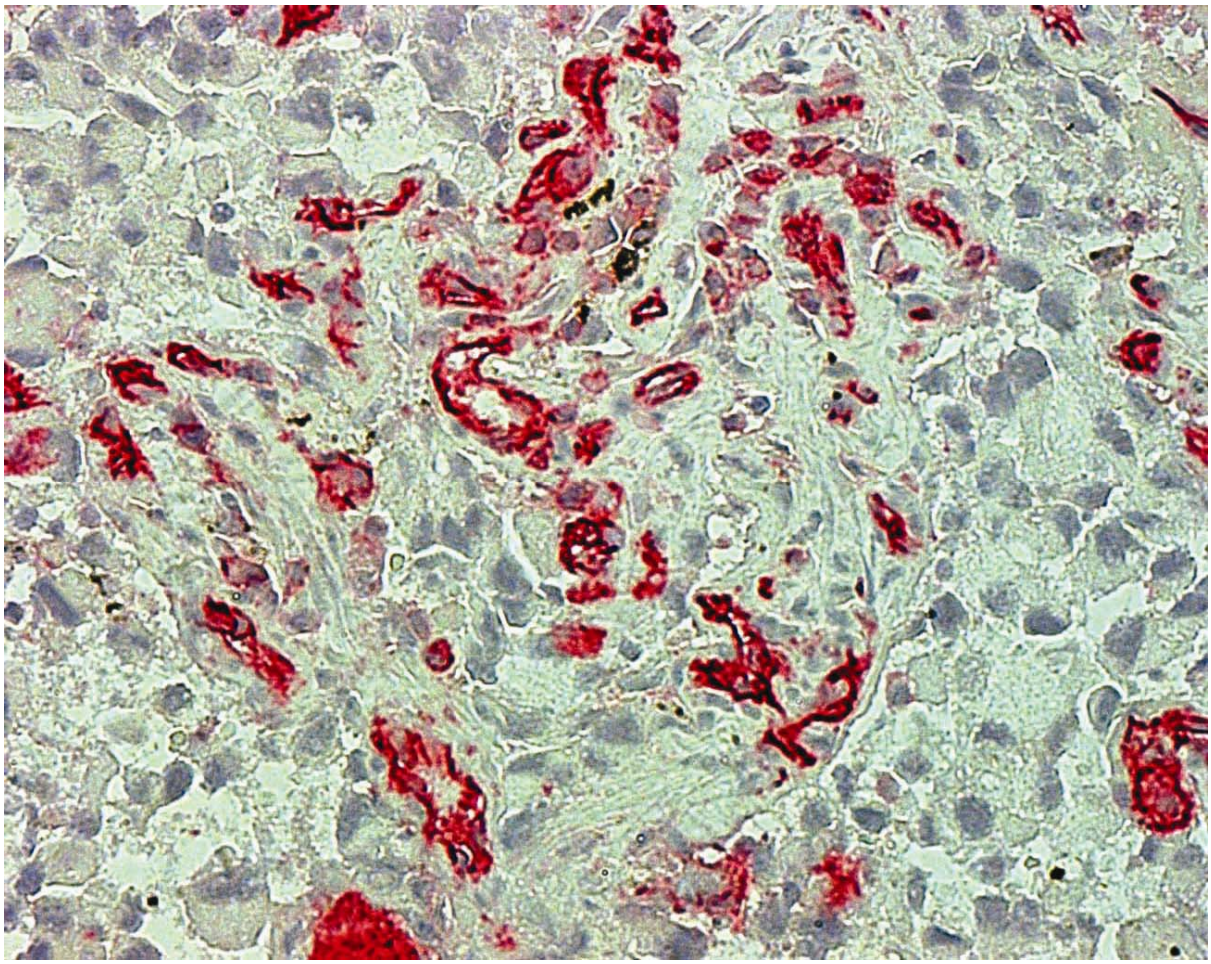
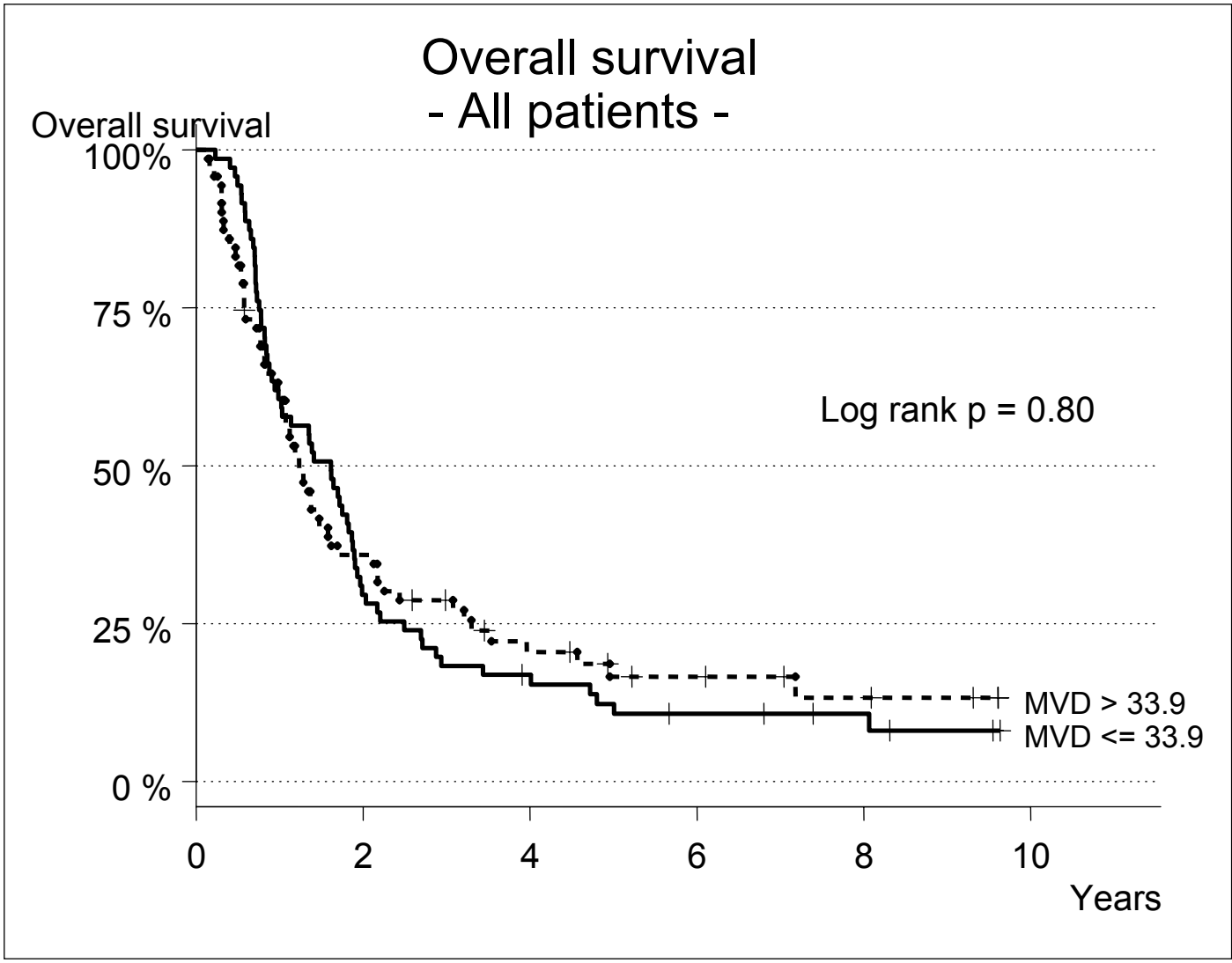


Figure 2: Kaplan-Meier estimates of overall (2a) and progression free survival (2b) of all analyzed patients (n = 142).


Overall survival
- All patients -
Overall survival
100%
75 %
50 %
25 %
0 %
Log rank p = 0.80
MVD > 33.9
MVD <= 33.9
0
2
4
6
8
10
Years

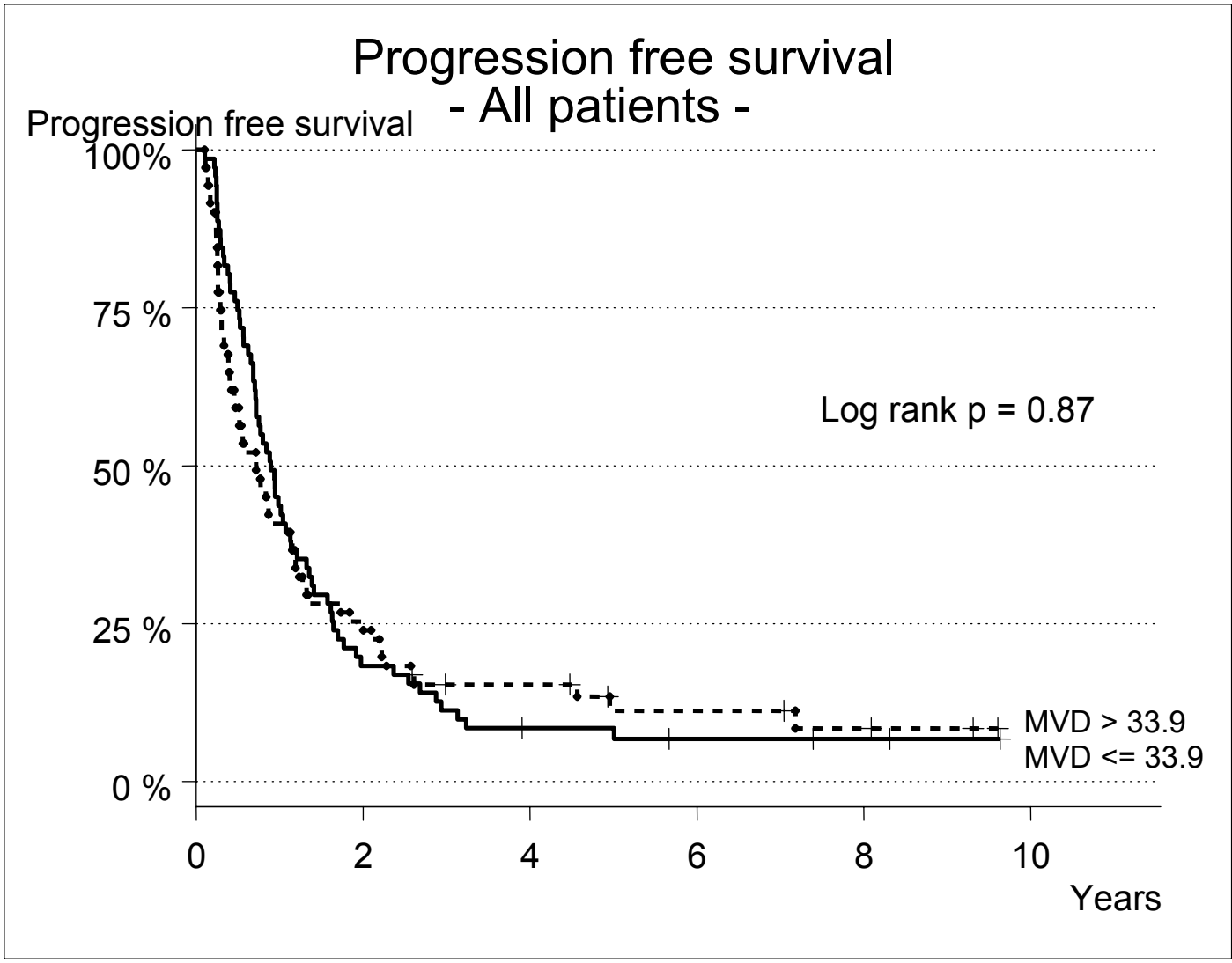
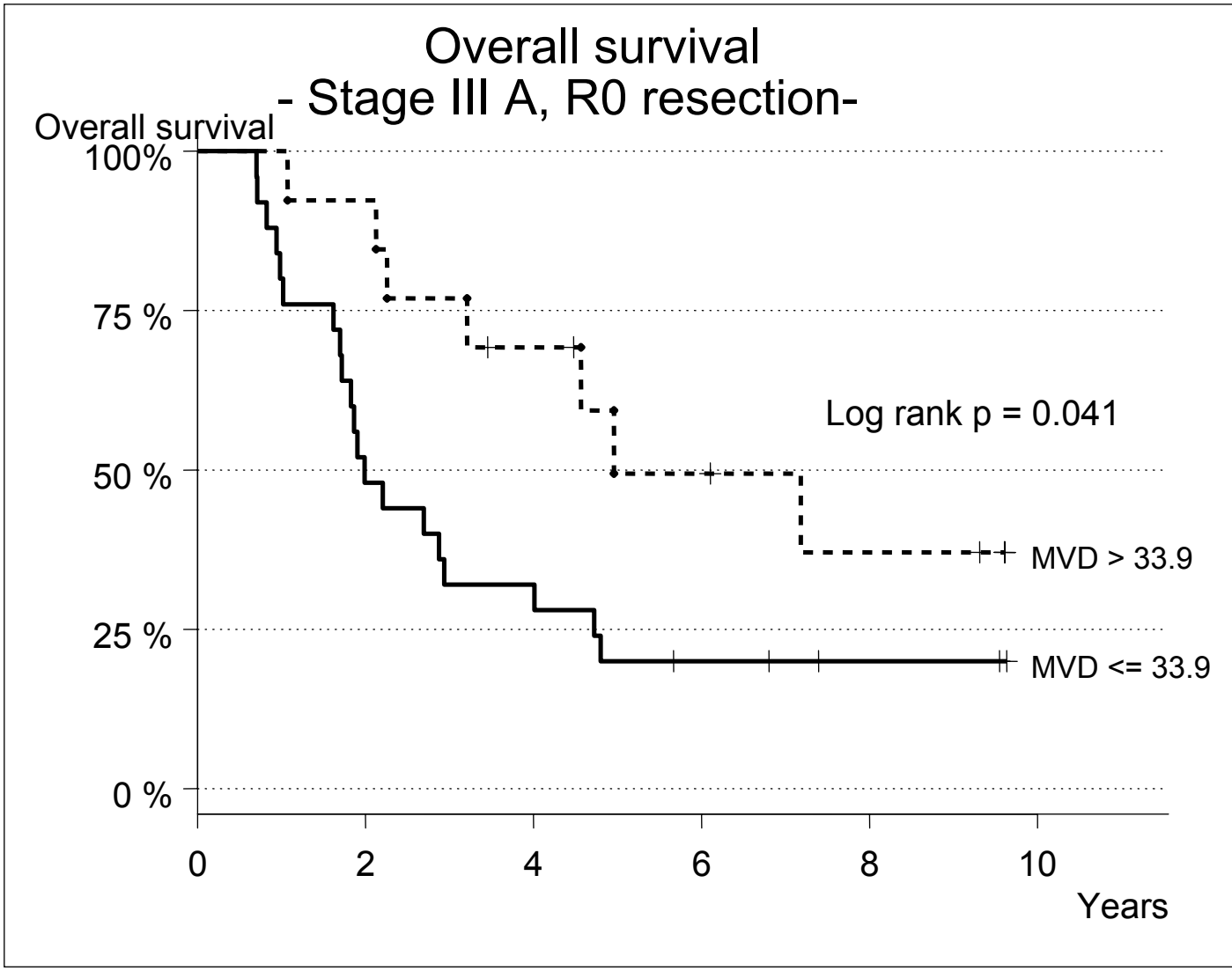
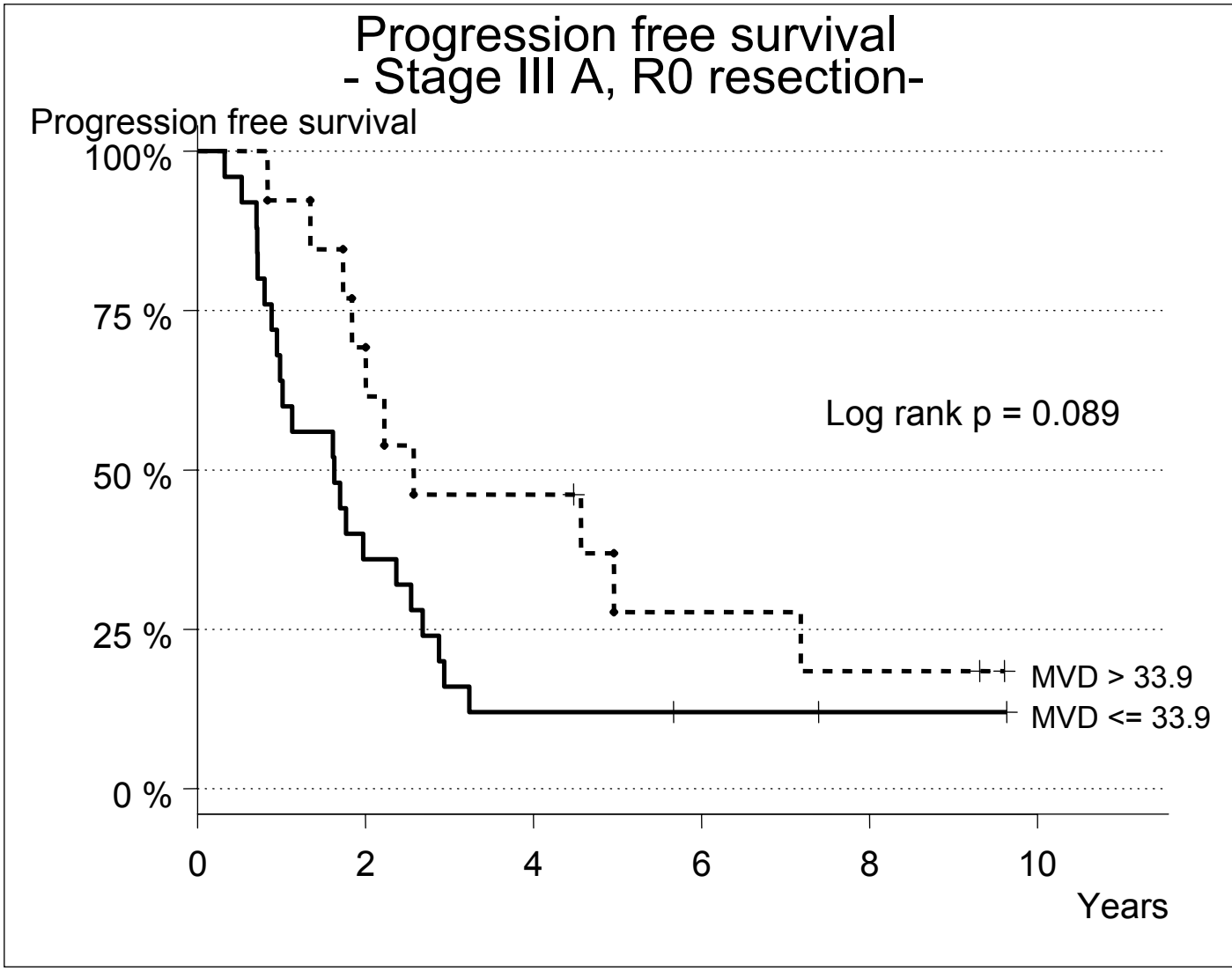


Figure 3: Kaplan-Meier estimates of overall (3a) and progression free (3b) survival of stage III A NSCLC patients with negative surgical resection margins grouped according to median MVD of 33.9 (n = 38). Overall survival was significantly prolonged in patients with high MVD compared to patients with low MVD (p=0.041).


Overall survival
- Stage III A, R0 resection-
Overall survival
100%
75 %
50 %
25 %
0 %
Log rank p = 0.041
MVD > 33.9
MVD <= 33.9
0
2
4
6
8
10
Years


Progression free survival
- Stage III A, R0 resection-
Progression free survival
100%
75 %
50 %
25 %
0 %
Log rank p = 0.089
MVD > 33.9
MVD <= 33.9
0
2
4
6
8
10
Years