High microvessel density determines a poor outcome in patients with diffuse large B-cell lymphoma treated with rituximab plus chemotherapy

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ABSTRACT

Background
Diffuse large B-cell lymphoma is a clinically and molecularly heterogeneous disease. Gene expression profiling studies have shown that the tumor microenvironment affects survival and that the angiogenesis-related signature is prognostically unfavorable. The contribution of histopathological microvessel density to survival in diffuse large B-cell lymphomas treated with immunochemotherapy remains unknown. The purpose of this study is to assess the prognostic impact of histopathological microvessel density in two independent series of patients with diffuse large B-cell lymphoma treated with immunochemotherapy.

Design and Methods
One hundred and forty-seven patients from the Leukemia Lymphoma Molecular Profiling Project (training series) and 118 patients from the Catalan Lymphoma-Study group-GELCAB (validation cohort) were included in the study. Microvessels were immunostained with CD31 and quantified with a computerized image analysis system. The stromal scores previously defined in 110 Leukemia Lymphoma Molecular Profiling Project cases were used to analyze correlations with microvessel density data.

Results
Microvessel density significantly correlated with the stromal score (r=0.3209; P<0.001). Patients with high microvessel density showed significantly poorer overall survival than those with low microvessel density both in the training series (4-year OS 54% *vs.* 78%; P=0.004) and in the validation cohort (57% *vs.* 81%; P=0.006). In multivariate analysis, in both groups high microvessel density was a statistically significant unfavorable prognostic factor independent of international prognostic index [training series: international prognostic index (relative risk 2.7; P=0.003); microvessel density (relative risk 1.96; P=0.002); validation cohort: international prognostic index (relative risk 4.74; P<0.001); microvessel density (relative risk 2.4; P=0.016)].

Conclusions
These findings highlight the impact of angiogenesis in the outcome of patients with diffuse large B-cell lymphoma and the interest of evaluating antiangiogenic drugs in clinical trials.

Key words: rituximab, aggressive non-Hodgkin's lymphoma, kidney, central nervous system.

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Introduction

Diffuse large B-cell lymphomas (DLBCL) are a heterogeneous group of tumors with different biological and clinical characteristics highlighted by the diverse clinical outcome of the patients. The addition of rituximab (R) has increased the survival of these patients by 10-15%,[1] but further risk stratification is necessary to improve the treatment strategies and outcome. Gene expression profiling (GEP) studies have shown that differences in the tumor microenvironment of DLBCL affect survival after treatment. A survival-predictor score, based on a multivariate model derived from the germinal center B-cell, stromal-1 and stromal-2 gene expression signatures, has the capability of predicting survival among patients with DLBCL treated with R-CHOP (rituximab plus cyclophosphamide, doxorubicin, vincristine and prednisone).[2] While the 'stromal-1-signature', related to extracellular matrix deposition and histiocytic infiltration is prognostically favorable, the angiogenesis-related signature ('stromal-2 signature') is associated with an unfavorable outcome.[2] GEP studies provide very robust prognostic information but are not routinely available for most patients. Several studies have provided increasing evidence that angiogenesis plays a role in the biology of lymphomas[3-8] and particularly in DLBCL.[9-11] The increasing attention given to new therapeutic strategies targeting angiogenesis in malignant neoplasias emphasizes the interest in assessing vascular density in the tumors to determine its prognostic relevance and biological impact. In the era of new anti-angiogenic therapies, a reliable stratification of patients with DLBCL according to the vascular density of their tumors may be useful to determine whether these new therapies might benefit all or only a subset of patients.

The purpose of our study was to evaluate whether the microvascular density in DLBCL quantified on tissues by a computer based imaging system has an impact on the outcome of these patients. This analysis was performed in two independent cohorts of patients treated with rituximab-based chemotherapeutic regimens.

Design and Methods

Clinical characteristics

A training group of 147 patients (79 males, 68 females; median age 64 years) with *de novo* DLBCL diagnosed between 1997 and 2007 was selected from the Leukemia Lymphoma Molecular Prophiling Project (LLMPP) consortium database based on the availability of formalin-fixed tissue material. Cases with transformed DLBCL, primary mediastinal large B-cell lymphoma (PMBCL), intravascular and primary effusion lymphomas, as well as immunodeficiency-associated tumors were excluded from the study. All patients were treated with rituximab-containing chemotherapy combinations (R-CT), in most cases, R-CHOP. Details of patients' main clinico-biological features, including therapy, are shown in Table 1. Median follow up of surviving patients was 3.7 years (range 0.13-11.3 years). Complete remission (CR) rate was 72%, whereas 4-year progression free survival (PFS) and overall survival (OS) were 56% and 66%, respectively. The definitions of CR, PFS and OS were based on standard criteria.[12]

An independent series of 118 patients (66 males, 52 females; median age 62 years) with *de novo* DLBCL diagnosed between 1999 and 2007 at the hospitals of the Catalan Lymphoma Study consortium (GELCAB) was used to validate the results. Thirty patients from the Hospital Clinic of Barcelona were initially included in the LLMPP studies and were not, therefore, considered in the GELCAB validation set. Median follow up of surviving patients was 3.85 years (range 0.12-9.7 years). The histological criteria to include the cases were the same as for the LLMPP patients. All the patients received R-CT. Main clinico-biological features of the patients included from the GELCAB series are listed in Table 1. Complete remission rate was 85.7% and 4-year PFS and OS were 63% and 68%, respectively. The list of the participating institutions from the LLMPP and the GELCAB consortiums are detailed in the appendices. Patients gave their informed consent according to the guidelines of the ethics committees involved.

Gene expression profiling (GEP)

Gene-expression profiling data from the LLMPP series were available in 110 cases. This information is accessible at *www.nci.nlm.nih.gov/geo/query/acc.cgi?token=rhojvaiekdsihqc&acc=GSE10846*, accession number GSE10846. Molecular subtyping of the DLBCL, according to the GEP signatures, included 47 germinal center B-cell (GCB) DLBCL, 53 activated B-cell (ABC) type DLBCL, and 10 unclassifiable cases. The previously defined gene expression stromal scores were used to analyze the correlations between GEP results and the microvasculature study data.

Immunohistochemistry and blood vessel density measurements

Tissue microarrays (TMAs) were constructed from pre-treatment biopsy specimens using duplicated cores of 1 mm per tumor sample. All cases were reviewed by a panel of experts in hematopathology. Samples were stained in an automated Bond® immunostainer with an antibody against CD31 (DAKO; 1/40 dilution, incubation of primary antibody for 30 min, antigen retrieval at pH 6 for 20 min). The microvessel density (MVD) was quantified using the digitalized images of the CD31-stained TMA cores (Figure 1) acquired with an Olympus BX51 microscope at x4 magnification and analyzed with an Olympus Cell B Basic Imaging Software. Microvessel areas were defined as vascular areas delineated by CD31+ staining. The MVD was calculated as the sum of all microvessel areas (μm^2) divided by the total area of the core

Table 1. Main clinical features of LLMPP and GELCAB cohorts.

	LLMPP N=147	GELCAB N=118
Sex (Male/Female)	79/68	66/52
Median age (range)	64 (22-91) years	62 (19-87) years
Ann Arbor stage	I-II= 47.4% (65) III-IV= 51.1% (70)	I-II=43.6% (51) III-IV=56.4% (66)
Extranodal sites		
0,1	87.8% (93)	90.4% (85)
≥2	12.2% (13)	9.6% (9)
IPI score		
0-1: Low risk	42% (60)	34.5% (39)
2-3: Low-intermediate risk High-intermediate risk	48.3% (69)	54.3% (50)
4-5: High risk	9.8% (14)	21.2% (24)
Treatment		
R-CHOP	80.5% (128)	91.6% (108)
R-CHOP-"like"*	19.5% (19)	8.4% (10)

**R-CHOP-like regimens - LLMPP series: R-CNOP (rituximab, cyclophosphamide, mitoxantrone, vincristine, prednisone), 12 cases; R-ESHAP (rituximab, etoposide, methylprednisolone, cytarabine, cis-platin), 3; R-CFM (rituximab, cyclophosphamide, fluorouracil, methotrexate), 4. GELCAB series: R-high dose-CHOP/R-ESHAP, 9 cases; R-CNOP, 1.*

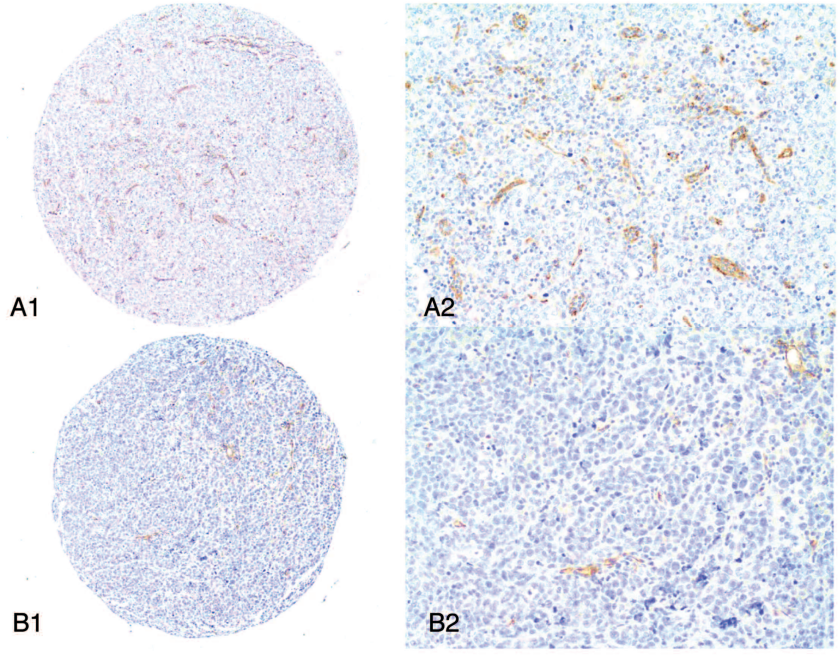

Figure 1. CD31 staining of DLBCL TMA cores. (A1, A2) High microvessel density and (B1, B2) low microvessel density. Image acquisition at x40 (A1, B1) and x100 (A2, B2) with an Olympus SC20 microscope.

analyzed (μm^2). MVD values were grouped in quartiles when necessary and considered high or low when above or below the 50th percentile respectively. TMAs were independently scored by 2 observers and discrepancies were resolved over a double-headed microscope. To determine whether the angiogenic values scored using TMA cores were representative of the tumor sample, in 40 cases microvessels were evaluated in whole tissue sections (WTS) and in TMA cores from the same tumor and compared by linear regression analysis. Three areas of the size of a TMA core rich in neoplastic cells were evaluated on each WTS for CD31+ MVD. The mean value of the three MVD measures in each WTS was compared to the MVD of the corresponding TMA core by linear regression analysis.

Statistical analysis

Categorical data were compared using Fisher's exact test with two-sided *P* values. For ordinal data, non-parametric tests were used. Spearman's correlation test was used to analyze the degree of linear association between MVD and the stromal scores or GEP data. The multivariate analysis of the variables predicting response was performed using a logistic regression analysis. The actuarial survival analysis was performed according to the Kaplan-Meier method, and the curves were compared by log rank test. The multivariate analysis for survival was performed with Cox's stepwise proportional hazards model.

Results

Results in the training series

Microvessel density (MVD)

The microvascular areas of the DLBCL cores showed great variability (Figure 1). The MVD values ranged between 0.77×10^{-3} and 92.7×10^{-3} (mean 14×10^{-3}). To determine whether the results obtained in the TMAs were representative of the whole tumor section (WTS), we studied the MVD in TMA cores and in the corresponding WTS of 40 cases. This comparison showed an excellent correlation between the MVD values in both types of samples (r=0.90; *P*<0.001).

Microvessel density and gene expression profiling

The stromal score had been previously defined as the component of the multivariate survival model composed by the difference between the stromal-2 and stromal-1 signature averages.[2] In the LLMPP DLBCL cohort, there was a significant correlation between MVD and stromal score (r=0.3209; *P*<0.001) (Figure 2). The entire set of the genes in the array platform was analyzed for statistical correlation with MVD with none of the genes showing a stronger correlation than that expected by chance. There was no statistically significant correlation between CD31 mRNA expression levels and the immunohistochemical CD31+ MVD.

DLBCL of the GCB type showed MVD mean values that were significantly lower than ABC type DLBCL ($11.8\pm12.2\times10^{-3}$ *vs.* $15.0\pm12.4\times10^{-3}$, respectively; *P*=0.05).

The main clinical features of the LLMPP series according to the MVD (low *vs.* high MVD) are listed in Table 2. Patients with advanced Ann Arbor stages showed higher MVD values than those with early stages (*P*=0.01). There was no correlation with other initial clinical characteristics. No significant differences were observed in terms of CR rates according to MVD values. Sixty-four patients eventually experienced failure to therapy or relapse with a 4-year PFS of 56% (95%CI: 47-65%). Patients with high MVD in the biopsy had shorter PFS than those with low MVD values (4-year PFS 48% *vs.* 65%, respectively; *P*=0.048) (Figure 3A). MVD and IPI had independent prognostic value for PFS (Table 3).

After a median follow up for surviving patients of four years, 46 patients had died, with a 4-year OS of 66% (95%CI: 58-74%). Patients with high MVD had a significantly poorer OS than those with low MVD (4-year OS: 54% *vs.* 78%, respectively; *P*=0.004) (Figure 3B). Other variables predicting poor OS were ABC-type DLBCL as assessed by GEP (*P*=0.001), poor performance status (*P*<0.001), and high serum LDH levels (*P*=0.001). As expected, IPI scores also showed prognostic impact for PFS (*P*<0.001) and OS (*P*<0.001). A multivariate analysis was performed, including IPI (low *vs.* intermediate *vs.*

high-risk) and MVD (low *vs.* high). In the final model, with 143 cases, both IPI (relative risk (RR) 2.7; *P*=0.003) and MVD (RR 1.96; *P*=0.002) maintained independent prognostic significance (Table 3). To analyze whether the prognostic interest of MVD was independent from molecular subtyping (GCB *vs.* ABC types), MVD (continuous variable) and molecular type were included in the model (n=100 cases), with both MVD (RR 1.057; *P*<0.001) and molecular subtype (RR 2.21; *P*=0.024) maintaining their prognostic value for OS. Finally, a multivariate analysis was performed including MVD (low *vs.* high) along with molecular type (GCB *vs.* ABC types) and IPI (low *vs.* intermediate *vs.* high-risk). In the final model, molecular subtype (RR 2.65; *P*=0.004) and IPI (RR 1.6; *P*=0.025) were the most important variables to predict OS.

Results in the validation cohort

One hundred and eighteen DLBCL patients of the GELCAB were used to validate the LLMPP results. In this series, MVD values ranged between 0.37 and 181×10-3 (mean 16×10-3). There was no significant difference between these values and those found in the training set. In the validation set, no correlation was observed between MVD and main clinical features at presentation. The 4-

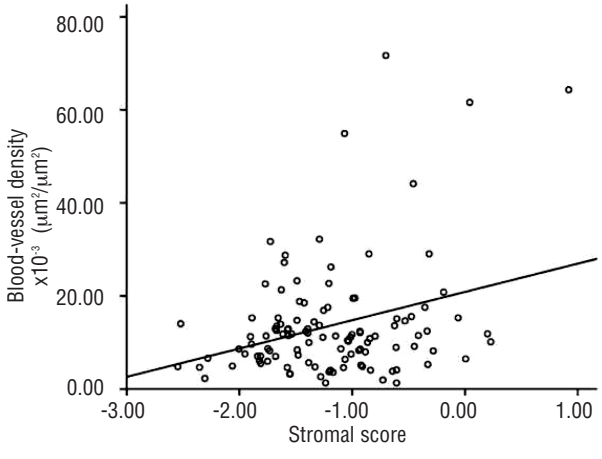

Figure 2. Spearman's correlation test. Linear regression analysis between microvessel density (MVD) and stromal score.

Table 2. Main clinical features and MVD.

	LLMPP (training cohort) N=147 MVD low	LLMPP (training cohort) N=118 MVD high	GELCAB (validation set) MVD low	GELCAB (validation set) MVD high
Median age (years)	64 (23-91)	64 (30-88)	64 (19-87)	61 (22-83)
Gender (M/F)	37/36	42/32	35/25	31/27
Advanced stage (III-IV) (%)	40	62	53	60
IPI (%)				
Low risk	51	33	39	31
L/I or H/I risk	42	55	44	44
High risk	7	12	17	25
CR rate (%)	79	66	88	83
4-year PFS (%)	65	48 *P*=0.048	72	54 *P*=0.03
4-year OS (%)	78	54 *P*=0.004	81	57 *P*=0.006

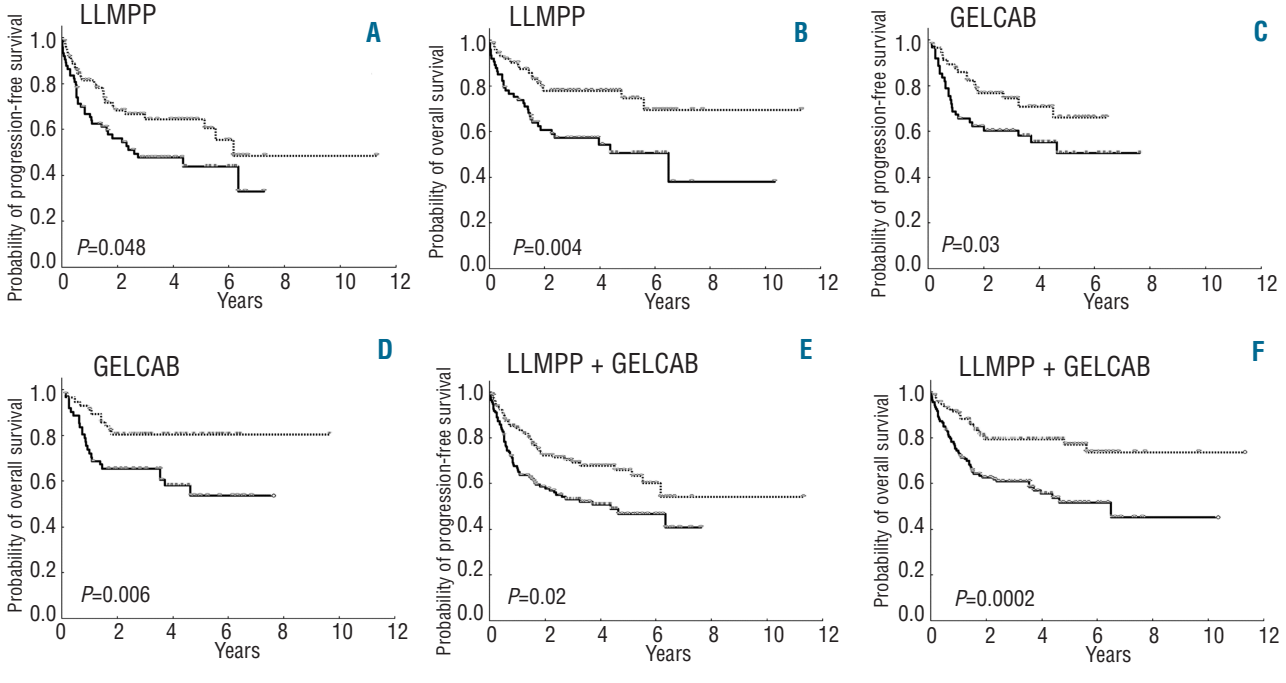

Figure 3. Kaplan-Meier survival analysis according to low and high microvessel density (MVD). (A) Progression-free survival (PFS) and (B) overall survival (OS) for the LLMPP series. (C) PFS and (D) OS for the GELCAB series. (E) PFS and (F) OS in merged analysis of entire series (LLMPP and GELCAB).

Table 3. Summary of multivariate analyses in LLMPP and GELCAB series.

	LLMPP (training cohort) N=147		GELCAB (validation set) N=118	
	P value	Relative risk	*P* value	Relative risk
Progression-free survival				
MVD (L *vs.* H)	0.01	1.6	0.05	1.89
IPI (L *vs.* I *vs.* H risk)	0.01	3.1	<0.001	3.7
Overall survival				
MVD (L *vs.* H)	0.002	1.96	0.016	2.4
IPI (L *vs.* I *vs.* H risk)	0.003	2.7	<0.001	4.74

MVD: microvessel density; IPI: International Prognostic Index; L: low; I: intermediate; H: high.

year PFS of the series was 62% (95%CI: 52-72%). Patients with high MVD in the biopsy (Table 2) had shorter PFS than those with low MVD values (4-year PFS 54 *vs.* 72%, respectively; *P*=0.03) (Figure 3C). After a median follow up for surviving patients of 3.85 years, 35 patients had died, with a 4-year OS of 68% (95%CI: 61-71%). Patients with high MVD showed significantly shorter OS in comparison to those with low MVD (4-year OS 57% *vs.* 81%, respectively; *P*=0.006) (Figure 3D). A multivariate analysis was performed for the training sample, including IPI (low vs. intermediate *vs.* high risk) and MVD (low *vs.* high). In the final model with 111 cases, both IPI (RR 4.74; *P*<0.001) and MVD (RR 2.4; *P*=0.016) maintained independent prognostic importance for OS (Table 3). Finally, the merged analysis of both series, training and validation set, including 265 patients, is shown in Figure 3E and F. Patients with high MVD had shorter PFS than those with low MVD values (4-year PFS 50% *vs.* 68%, respectively; *P*=0.003) (Figure 3E). Patients with high MVD showed significantly shorter OS in comparison to those with low MVD (4-year OS 55% *vs.* 79%, respectively; *P*=0.0001) (Figure 3F).

Discussion

In this study, we have shown that differences in the blood vessel density of DLBCLs have a consistent relationship with the outcome of patients treated with R-CT. Patients with a high MVD show a significantly poorer PFS and OS than those with a low MVD. These results were evident in two independent series of DLBCL patients treated with R-CT and maintained their independent prognostic value in a multivariate analysis. Thus, an increased MVD was able to discriminate patients with poor-risk DLBCL independently of the IPI risk groups. The possibility of recognizing patients who have a significantly worse prognosis due to a highly vascularized tumor can be useful in the light of the many new antiangiogenic therapies which are now available and which can be incorporated into clinical trials.

Increased MVD has also been described as a negative prognostic factor in solid tumors but has not been explored in DLBCL treated with R-CT.[13] The prognostic value of the MVD in our study agrees with previously published GEP data on DLBCL.[2] The gene expression signatures 'stromal 1' and 'stromal 2', related to extracellular matrix and angiogenesis-related genes, respectively, were synergistic in predicting survival. These two signatures were combined into the stromal score and high values of this score predicted an adverse clinical outcome.[2] Interestingly, the MVD measured in our study showed a statistically significant correlation with the GEP global 'stromal score' but not with the individual angiogenesis-related 'stromal-2' signature nor with the CD-31 mRNA expression levels of each case. The fact that there is no relationship between the MVD and the 'stromal-2' signature, which includes CD-31 mRNA expression amongst other genes specifically expressed in the endothelium, suggests that vascular density is not just a direct surrogate of high expression levels of angiogenesis-associated genes. It may reflect an integration of the interplay between angiogenesis-related genes and genes of the 'stromal-1' signature involved in the remodeling of the extracellular matrix that modulate the process of neoangiogenesis in tumors. In fact, the new blood vessels formed in the tumor stroma tend to be highly irregular and tortuous.[14,15] The MVD measured in this study may capture these morphological features of neoangiogenesis. These findings support the idea that MVD may be a surrogate biomarker of the GEP 'stromal score' in DLBCL and may help to stratify these patients according to the biological risk associated with angiogenesis.

In our study, the ABC-type DLBCLs showed a higher MVD than tumors with a GCB profile. The ABC-type DLBCL arises from a post-germinal center B cell that is blocked during plasmacytic differentiation, whereas the GCB-type DLBCL subtype arises from a germinal center B cell. These two types of DLBCL also differ in their profile of genetic alterations and dysregulation of molecular pathways.[16-20] In particular, the ABC type DLBCL shows constitutive activation of NF-kB that may be related to the presence of mutations in multiple genes regulating this pathway.[21-24] The NF-kB transcription factor has been associated with multiple aspects of angiogenesis by regulating several genes involved in this process such as VEGF, IL-8 and several metalloproteinases, among others.[25-27] Therefore, the higher MVD observed in the ABC-type DLBCL is concordant with the NFκB activation of these tumors.

Previous reports have focused on the prognostic value of angiogenesis in DLBCLs.[9,10] In those studies, microvessel density was evaluated in a semi-quantitative manner and the immunohistochemical markers differed among studies.[3,9,10] Although different methods were used for evaluation, these papers suggest that both angiogenesis and lymphangiogenesis play a role in lymphomas, including DLBCL.[7,8,10,11,28]

We approached the evaluation of angiogenesis in DLBCL using a computerized imaging system. We chose CD31, the platelet adhesion molecule PECAM1, for the immunohistochemical evaluation of the microvessels, since it is one of the genes included in the stromal-2 signature and because it is expressed both on the vascular and lymphatic endothelium as opposed to other markers such as CD34 or FVIII, that are mostly expressed in the vascular endothelium.[29] In our study, we have seen that the total microvascular area rather than the number of vessels per area determines the outcome of our patients. This approach captures the biological significance of angiogenesis in DLBCLs and agrees with experimental studies suggesting that the function of the vasculature is more important than the vessel count.[15,30]

In conclusion, the biological insights gained from this study provide a new perspective for future clinical trials incorporating antiangiogenics. On the basis of the results of both the previous GEP study and the current immunohistochemical MVD analysis, it is possible to suggest that the new antiangiogenic therapies may benefit in particular a subgroup of patients with aggressive DLBCL characterized by a high tumor blood vessel density.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

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