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Prognostic impact of CD31-positive microvessel density in follicular lymphoma patients treated with immunochemotherapy

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ABSTRACT

Background: Tumour-infiltrating mast cells (MCs) can remodel tumour microenvironment and growth by suppressing immune responses and potentiating angiogenesis. Furthermore, accumulation of MCs in follicular lymphoma (FL) correlates with unfavourable prognosis after immunochemotherapy. Here we investigated whether tumour vascularity is associated with MC content and outcome in FL patients treated with immunochemotherapy.

Patients and methods: Microvessel density (MVD) and MC content were determined immunohistochemically from pretreatment samples of 95 FL patients using CD31, CD34 and mast cell tryptase antibodies. Gene expression data from a separate set of 24 FL patients were analysed for comparison. All patients were treated with the combination of rituximab (R) and cyclophoshamide-doxorubicin–vincristine–prednisone (CHOP) chemotherapy.

Results: Increased CD31+ MVD correlated positively with the number of tumour infiltrating MCs and CD34+ vessels, and negatively with the outcome. Overall survival and progression-free survival were significantly better among patients with low CD31+ MVDs. In multivariate analyses, CD31+ MVD had prognostic value independently of Follicular Lymphoma Prognostic Index but not of MC content. Consistent with the immunohistochemical data, high CD31/*PECAM1* mRNA levels were associated with adverse outcome. Conversely, a positive prognostic impact of *VEGF* mRNA expression on the outcome was found.

Conclusion: Vascularity is associated with MC content and outcome in R-CHOP-treated FL patients.

1. Introduction

Follicular lymphoma (FL) is the second most common subtype of non-Hodgkin lymphomas (NHLs). It is an indolent disease characterised by frequent relapses, and considered incurable in most cases due to the gradually developing resistance to therapy or transformation into more aggressive lymphoma.[1]

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In FL, the composition of the non-malignant lymphoma microenvironment contributes significantly to the clinical behaviour of the disease. In particular, tumour-infiltrating regulatory T-cells, follicular dendritic cells, macrophages and mast cells (MCs) seem to have prognostic impact on FL.[2–7] Of note, however, the prognostic significance of the tumour microenvironment appears to be highly dependent on a given therapy.[8]

Angiogenesis has a growth-promoting role in various solid tumours, and there is an increasing evidence of its importance also in the field of haematolymphoid malignancies.[9] On the other hand, cytokines and chemokines released from the activated immune cells, like MCs and macrophages promote tumour cell growth and survival along with angiogenesis.

We have previously demonstrated that tumour-infiltrating MCs have an unfavourable impact on the prognosis of R-CHOP-treated FL patients.[6] In the present study, we have addressed the possible association between the number of tumour infiltrating MCs and vascularisation, and further elucidated the prognostic implication of tumour angiogenesis in FL patients uniformly treated with R-CHOP regimen.

2. Patients and methods

2.1. *Patients and samples*

This is a population-based retrospective analysis of FL patients treated with a combination of rituximab (R) and cyclophosphamide, doxorubicin, vincristine and prednisone (CHOP) regimen. Initially, study population consisted of 95 FL patients. The patients were eligible if they had received R-CHOP as a first-line treatment, if paraffin-embedded lymphoma tissue was available for immunohistochemical stainings and if the sample had been taken before R-CHOP treatment. All patients had a clinical indication for treatment, and were sequentially treated at the Helsinki University Central Hospital (HUCH) or Kuopio University Hospital during 1999–2005. The baseline clinical characteristics were collected, and the risk stratification was performed according to the Follicular Lymphoma International Prognostic Index (FLIPI).[10] For validation, a previously generated microarray data set[11] with updated follow-up was used. The data set contains gene expression profiles analysed with Agilent Human IA oligonucleotide microarrays from the lymphoma tissues of 24 R-CHOP-treated FL patients. Raw expression microarray data are available at ArrayExpress archive (http://www.ebi.ac.uk/microarray-as/ae/; ID: E-MEXP-2305). The protocol and sampling were approved by Ethical Committee, Institutional Review Board and Finnish National Authority for Medicolegal Affairs.

2.2. *Immunohistochemistry and microvessel density counting*

Immunohistochemistry for CD31 (1:200, Novocastra Laboratories Ltd., Newcastle upon Tyne, UK) was performed on formalin-fixed, paraffin-embedded tissue sections either on individual slides ($n = 48$) or as a part of tissue microarray (TMA; $n = 47$). Briefly, the sections were deparaffinised with xylene and rehydrated in graded alcohol, treated in an autoclave in 10 mmol/l sodium citrate (pH 5.0) for 2 min and washed with phosphate buffered saline. Primary antibody was incubated at 4 °C overnight, and antibody binding was detected by Vectastain ABC kit reagents (Vector Laboratories, Burlingame, CA, USA). MC tryptase staining was performed on whole tissue sections as described earlier.[6] MVD was quantified as a number of CD31+ microvessels defined as distinct lumen containing vascular structures per high power field at ×250 (field of view 0.407 mm^2, including the whole TMA core) using a Leitz Laborlux 12 bright-field microscope (Leitz Wetzlar GmbH). For TMA, at least three representative

Table 1 – Relationship of CD31+ MVD with patient and baseline characteristics.

Characteristic	All patients $n = 95$ (%)	Low CD31+ MVD (⩽lowest tertile) $n = 32$	High CD31+ MVD (>lowest tertile) $n = 63$	*p*
MVD count median (range)	21.5 (4–74)	9 (4–19)	29 (16–74)	NA
Gender				
Female	53 (56)	25 (76)	28 (45)	0.005
Male	42 (44)	8 (24)	34 (55)	
Age				
⩽60	60 (63)	21 (64)	39 (63)	1.000
>60	35 (37)	12 (36)	23 (37)	
Grade				
I	51 (54)	19 (58)	32 (52)	0.937
II	28 (29)	10 (30)	18 (29)	
III	13 (14)	4 (12)	9 (14)	
Missing	3 (3)	0 (0)	3 (5)	
FLIPI				
0–2	58 (61)	22 (67)	36 (58)	0.226
3–5	33 (35)	9 (27)	24 (39)	
Missing	4 (4)	2 (6)	2 (3)	

MVD, microvessel density; NA (not applicable); FLIPI, Follicular Lymphoma International Prognostic Index.

cores were available in 61% of cases. With sections, three fields with the highest density of vessels were counted and an average of two highest scores was reported. CD31+ sinusoidal structures were avoided. Because of the variation in the sample size between TMA and whole tissue sections, and a greater possibility to include hotspots in whole tissue sections, cut off points were analysed separately. MVD counts denoted as 'high' are above the lowest tertile (score of 15 for TMA, range 4–42, and 20 for whole sections, range 5–74), and those denoted as 'low' are below that. Scoring was performed in a blinded manner. To confirm the data on CD31 stainings, 55 whole tissue sections were stained with anti-CD34 (1:100, clone QBEnd 10, Dako Denmark A/S, Glostrup, Denmark). The specimens were analysed independently by two investigators (M.T. and P.B.). Reproducibility of the scoring method was investigated by reanalysing 30 randomly chosen

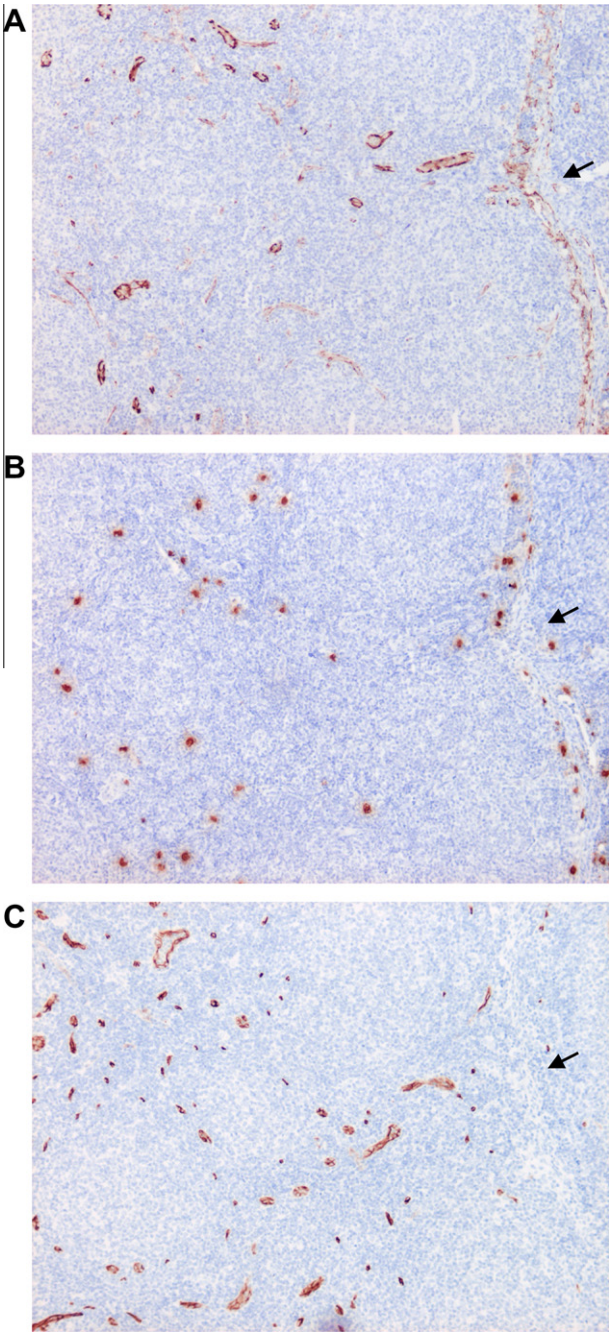

Fig. 1 – Microvessel density is associated with MC content in patients with FL. (A) Microvessels stained with anti-CD31 antibody (100×). (B) MCs co-localise with vessels (anti-MC tryptase, 100×). (C) Microvessels stained with anti-CD34 antibody (100×). Arrows indicate the CD31+ and CD34– sinuses.

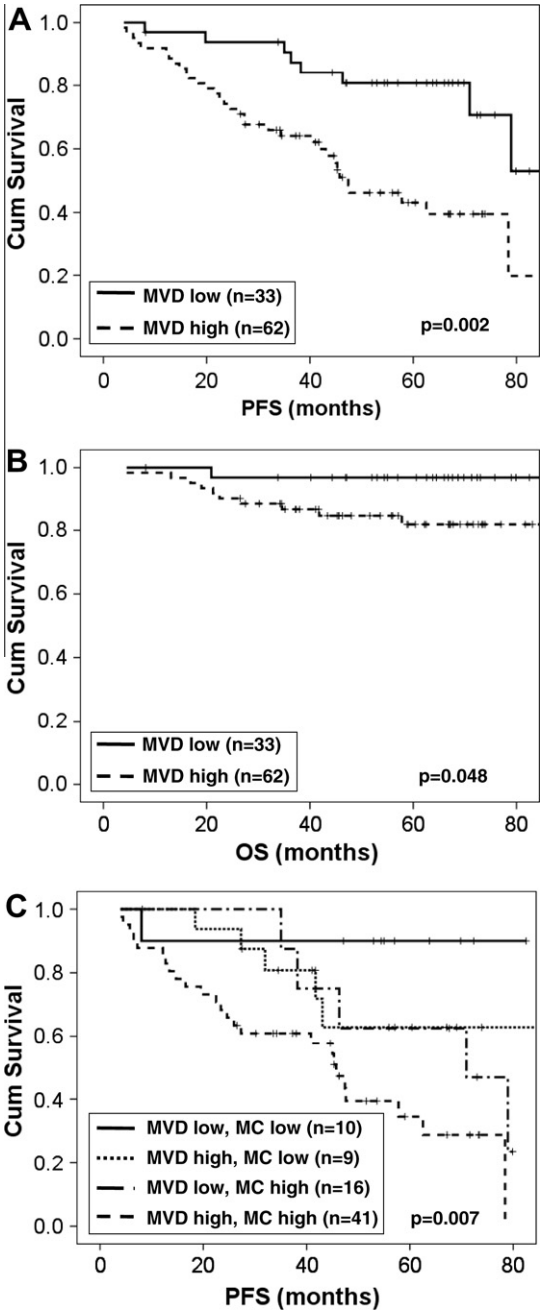

Fig. 2 – The outcome of R-CHOP-treated FL patients according to CD31+ MVD. Kaplan–Meier analysis shows poor PFS ($p = 0.002$) (A) and OS ($p = 0.048$) (B) with increasing CD31+ MVD. (C) The patients with both low MC and MVD counts have improved PFS as compared to the groups of patients with high MC and/or CD31+ MVD counts ($p = 0.007$).

samples. The coefficient of variation for intraobserver and interobserver variabilities was 10% and 15%, respectively.

2.3. Statistical analyses

The strength of the associations between MC content and MVD, and CD31+ and CD34+ vessels was analysed with Spearman correlation coefficient. Chi square test was performed to evaluate the differences in the frequency of the prognostic factors. Survival rates were estimated by the Kaplan–Meier method and the differences were compared by the log rank test. Cox uni- and multivariate analyses were used to test the prognostic impact of identified factors on OS and PFS. Probability values below 0.05 were considered statistically significant. All p-values were two-tailed. They were not corrected for multiple testing, as it was the intent of these results to be hypothesis generating.

3. Results

3.1. Association between MVD and MC content

The clinical characteristics of the patients are listed in Table 1. The median age of the patient cohort was 57 years (range 27–82 years). The median follow-up time from the start of the therapy was 64 months (range 8–100 months). When the patients were further divided into two groups based on the CD31+ MVD counts, no differences in age, grade or FLIPI scores were observed. Instead, low MVD group contained significantly more females.

In immunohistochemical stainings a typical pattern of distribution of blood vessels was detected, showing prominent vascularisation in the interfollicular areas and only a few vessels within the follicles (Fig. 1A). Therefore, the counted hot spots were also located between the follicles.

It could be evidently seen that MCs co-localised with CD31+ vessels (Fig. 1A and B). Furthermore, positive correlation between MC count and CD31+ MVD was found ($r_s = 0.260$, $p = 0.023$, $n = 76$).

To confirm the data on CD31 stainings, a subset of 55 samples was stained and evaluated for CD34+ vessels (Fig. 1C). Although the median number of CD34+ vessels was greater than that of CD31+ ones (medians 56.3 versus 34.3 on the corresponding whole tissue sections), a subsequent comparison of MVDs demonstrated a borderline correlation between CD31+ and CD34+ vessels ($r_s = 0.341$; $p = 0.056$, $n = 32$). Furthermore, a statistically significant correlation between MCs and CD34+ vessels was found ($r_s = 0.314$, $p = 0.022$, $n = 53$).

3.2. Survival analyses

In Cox univariate analysis, CD31+ MVD had prognostic impact on PFS as a continuous variable ($p < 0.001$), and a borderline significant impact on OS ($p = 0.058$). When CD31+ MVD count (low versus high) was entered into a multivariate analysis with FLIPI, it had negative prognostic value on PFS independently of FLIPI (RR 2.36; 95% CI 1.098–5.051, $p = 0.028$). However, the prognostic value was not independent on MC content (RR 2.43; 95% CI 0.963–6.138, $p = 0.060$).

Five-year PFS for the whole patient cohort was 57%. Because the survival curves of the patients with intermediate and high CD31+ MVD counts overlapped (both in TMA and whole tissue sections), but diverged remarkably from the patients in the lowest tertile, the lowest tertile was chosen to be the cut-off value. According to Kaplan–Meier estimates, FL patients in the high CD31+ MVD group (>lowest tertile) had a significantly worse outcome than the ones in the low group (5-year PFS, 43% versus 81%, $p = 0.002$; 5-year OS, 82% versus 97%, $p = 0.048$; Fig. 2A and B). Prognostic impact was especially seen in the patients with low FLIPI scores ($p = 0.008$). When the CD31+ MVD-associated outcome was adjusted to gender, the difference in survival was seen in both females and males ($p = 0.009$). If the median was used as a cut-off value, a significant difference in the PFS, and a trend in OS between high and low CD31+ MVD groups were also observed (5-year PFS 39% versus 72%, $p = 0.013$, 5-year OS 83% versus 91%, $p = 0.225$). Furthermore, if absolute CD31+ MVD counts from whole tissue sections and TMAs were pooled together, and the patients subsequently divided into high and low subgroups, high CD31+ MVD (>lowest tertile) remained as a significant adverse prognostic factor for survival (data not shown). When the patients were further divided into four groups in accordance with the MC content, the patients with both high CD31+ MVD and high MC counts had a very poor outcome. Conversely, the group with low CD31+ MVD and MC counts had an exceptionally good prognosis (5-year PFS 35% versus 90%, $p = 0.007$) (Fig. 2C). In comparison, CD34+ MVD was not associated with survival (univariate $p = 0.417$ for PFS and $p = 0.936$ for OS).

In order to find support for the CD31 immunohistochemical data, we analysed the prognostic significance of *CD31/PECAM-1* and other genes involved in angiogenesis from our previous microarray data set.[11] The clinical characteristics of these patients are shown in Table 2. With the updated follow-up of 73 months (8–100 months), the 5-year PFS rates for the whole cohort and primary treated patients were 44% and 63%, respectively. In comparison with MVD cohort, no significant differences in baseline characteristics or PFS rates were observed ($p > 0.2$). Consistent with the MVD data, high *PECAM1* mRNA levels were associated with adverse outcome. The 5-year PFS for the patients with high *PECAM-1* mRNA levels (>median) was 82% compared with 8% of those with lower

Table 2 – Patient characteristics of the microarray group.

	Microarray group ($n = 24$)
Median age (years)	53 (38–77)
Female/male (%)	62/38
FLIPI (%)	
0–2	54
3–5	46
Grade (%)	
I	58
II	33
III	9
Primary disease (%)	71

FLIPI, Follicular lymphoma International Prognostic Index.

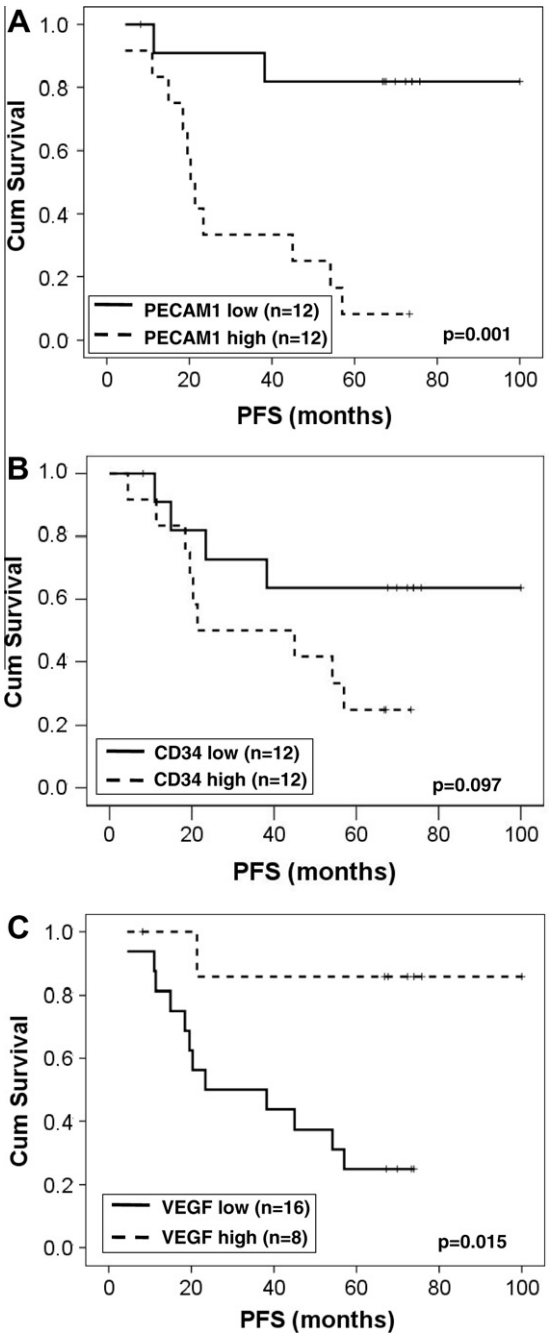

Fig. 3 – The outcome of R-CHOP-treated FL patients according to *PECAM1*, *CD34* and *VEGF* transcripts. (A) High *PECAM1* mRNA expression predicts poor PFS ($p = 0.001$). (B) High *CD34* expression is marginally significant predictor of worse PFS ($p = 0.097$). (C) High *VEGF* expression is associated with improved PFS ($p = 0.015$).

levels (⩽median, $p = 0.001$; Fig. 3A). In addition, a non-significant trend towards poorer PFS was observed in patients with high (>median) *CD34* mRNA levels (5-year PFS 64% versus 25%, $p = 0.097$; Fig. 3B). Other angiogenic factors with prognostic impact on PFS in univariate analysis were also identified (Table 3). Interestingly, high *VEGF* expression (>highest tertile) predicted better survival when compared to lower *VEGF* mRNA levels (5-year PFS 86% versus 25%, $p = 0.015$; Fig. 3C). *PECAM1* mRNA expression also correlated with CD34 ($r_s = 0.566$, $p < 0.004$) and VEGF ($r_s = -0.503$, $p = 0.012$) expression levels.

Table 3 – Correlation of angiogenic gene expression with prognosis as measured by the patient outcome predictors.

Gene	Significance	
	t-test (*p*)	Cox regression (*p*)
PECAM1	0.006	0.014
CD34	0.052	0.055
VEGF	0.006	0.032
VEGFB	0.057	0.029
VEGFC	0.081	0.147
FLT1	0.232	0.465
KDR	0.456	0.409
FLT4	0.028	0.053
ANGPT2	0.547	0.366
PGF	0.746	0.385
MMP9	0.067	0.027

t-test, comparison of relapsed patients versus patients in remission.

4. Discussion

In the present study, we demonstrate that CD31+ MVD and angiogenic factors, such as VEGF have prognostic impact on the survival of R-CHOP-treated FL patients. Immunohistochemically defined low CD31+ MVD allowed us to select a subgroup of FL patients with favourable prognosis. Gene expression analysis of an independent patient cohort supported our immunohistochemical findings. Together, the data showing that CD31+ MVD and expression of angiogenic factors predict outcome both at protein and at mRNA levels and in two independent patient cohorts encourage us to believe that a novel biological predictor of outcome for immunochemotherapy-treated FL patients has been identified.

Our patient samples consisted of approximately 50% whole tissue sections and 50% TMA cores. Because CD31+ hot spots were located in interfollicular areas, TMAs were re-evaluated to select the cores mainly consisting of interfollicular areas. Considering that CD31 expression can also be observed in macrophages, care was taken to count only lumen containing vascular structures.[12] Furthermore, since CD31 in the lymph nodes is expressed not only in capillaries and venules but also in specific dendritic cells lining the sinuses,[13] the results were confirmed with CD34 stainings. When the CD31 and CD34 stainings were compared, the overall microscopic view was quite identical, apart from some residual sinuses (arrows in Fig. 1A–C). Cases with nodular sinuses were re-evaluated, and counting was performed avoiding sinusoidal structures. In accordance with the results by Norrby and colleagues, CD34 staining highlighted clearly more vessels than CD31.[14] However, CD34+ MVD was not associated with survival, whereas CD31+ vessels had prognostic impact on survival also in this smaller cohort of patients (data not shown). The reason for the difference is

currently unclear but it may be associated with increased angiogenic sprouting, which can only be highlighted with CD31 but not with CD34, and has been recently associated with poor prognosis in FL.[15]

Our results further suggest that MCs contribute to angiogenesis in FL. There is a growing acceptance of the active role of myeloid cells in tumour growth and angiogenesis.[16,17] MCs are often found in close association with blood vessels. They secrete a number of pro-angiogenic molecules, and drive neovascularisation *in vitro*. The correlation of MC density with angiogenesis in immunochemotherapy-treated FL patients is in agreement with the findings of Ribatti and colleagues,[18] who demonstrated association between MCs, angiogenesis and clinically progressive disease in a heterogeneous population of B-cell NHLs. Likewise, Gratzinger and colleagues[19] showed a negative association with CD34+ MVD and survival in chemotherapy treated diffuse large B-cell lymphoma (DLBCL) patients. Consistent with our data, they further observed a trend of positive prognostic impact of VEGF expression on the outcome of DLBCL patients.[19] However, in their subsequent study for R-CHOP-treated DLBCL, no association between CD34+ MVD or VEGF expression and outcome was observed.[20] Jorgensen and colleagues[21] studied MVD in different lymphoma subtypes, and found that high MVD located interfollicularly was associated with poor OS in FL. In contrast, Koster and colleagues[22] have reported that increased vascularisation in FL has favourable impact on survival. This difference might result from the fact that in the Koster's study, all patients received interferon-α2b, which is a known antiangiogenic agent. Nonetheless, several studies indicate the prognostic importance of angiogenesis on the outcome of lymphoma patients. Most of the studies have focused on the differences in vasculature and survival between histological subtypes. In addition, the patients have had heterogeneous treatment regimens, and only one has had rituximab-containing therapies. Thus, our results on uniformly R-CHOP-treated FL patients not only confirm previous findings but also suggest further that addition of rituximab to chemotherapy does not interfere with the CD31-positive MVD (or VEGF) associated outcome.

Preliminary clinical studies on antiangiogenic therapies have reported encouraging results, and the growing list of antiangiogenic drugs is in various stages of development.[23,24] The recent work with targeted immunotherapy to the sites of tumoural neovasculature in a mouse model of human B-cell lymphoma also highlights the importance of angiogenesis in lymphomas.[25] Our findings support the therapeutic efforts against the sites of angiogenesis in haematologic malignancies also in the rituximab era.

In conclusion, we have demonstrated that CD31+ MVD is associated with MC content and adverse outcome in R-CHOP-treated FL patients. The gene expression data support these findings. Together, the results provide further rationale for testing antiangiogenic agents in combination with immunochemotherapy in FL patients.

Conflict of interest statement

None declared.

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