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New Concept of the Role of Angiogenesis in the Pathogenesis of Chronic Lymphoproliferative Diseases

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ABSTRACT

Background: The importance of angiogenesis for the progressive growth and viability of solid tumours is well established, while little is known about angiogenesis in leukemia. Recent studies suggested that angiogenesis may be involved in hematological malignancies.

Aim of the Work: The aim of this study was to evaluate the role of angiogenesis and its significance in the pathogenesis of lymphoproliferative neoplasms.

Patients and Methods: The study included 26 patients with chronic lymphoproliferative disorders, 20 with non-Hodgkin lymphoma, and 6 patients with CLL. The study also included 10 lymphoma patients with normal bone marrow biopsy as control group. Immunohistochemical staining of bone marrow blood vessels using anti-vWF, anti-thrombomodulin and VEGF expression in bone marrow trephine biopsy sections were done to all patients and controls.

Results: The number of bone marrow blood vessels per high power field when using anti vWF was significantly higher in cases of follicular lymphoma (FL) (p<0.001), diffuse large cell lymphoma (DLCL) (p<0.001) and chronic lymphocytic leukemia (CLL) (p<0.001) with median and inter-quartile range (IQR) of 11.5 (2.75), 12.0 (1.75) and 10.0 (2.0) respectively as compared with the control group 2.5 (1.5).

On using anti-thrombomodulin as endothelial cell marker, bone marrow blood vessels per high power filed was significantly higher in FL (p<0.001), DLCL (p<0.001) and CLL (p<0.001) with median and IQR of 12.0 (2.5), 11.0 (2.75) and 9.5 (3.5) respectively as compared with the control group with a median of 3.0 (2.0).

There was a positive correlation between the number of bone marrow blood vessels counted using immunohistochemical staining with anti-vWF and anti thrombomodulin antibodies as endothelial cell markers.

Vascular endothelial growth factor (VEGF) expression (intensity of the reaction and percentage of positive cells) was statistically higher in cases of FL (p<0.001), DLCL (p<0.001) and CLL (p<0.001) with a median IQR of 40 (17.5), 40.0 (10.0) and 35.0 (20.0) respectively when compared with the control group 4.0 (4.0). Immunoreactive score (IRS) was also increased in FL, DLCL and CLL as compared to the control group. There was also a positive correlation between VEGF expression and the number of bone marrow blood vessels counted by immunohistochemical staining with anti-vWF and antithrombomodulin antibodies.

Conclusion: The increase of bone marrow blood vessels, which is measured by immunohistochemical staining with anti-vWF and antithrombomodulin, and the increased expression of VEGF suggests that increased angiogenesis may play a role in the pathogenesis of the disease.

Key Words: *CLPD – NHL – Anti-vWF – Thrombomodulin – VEGF.*

INTRODUCTION

Lymphoproliferative neoplasms are a group of clonal diseases that arise as a result of somatic mutation in lymphocyte progenitor. The progeny of the affected cell carry the phenotype of a B, T or natural killer cell as judged by immunophenotyping **[1]**. According to the National Cancer Institute of Cairo, lymphoma and leukemia constitute 12% of all cancers **[2]**. In 2008 WHO classified lympho-proliferative malignancies as B and T cell disorders **[3]**.

General clinical features of chronic lymphoproliferative disorders (CLPD) include fever, malaise, weight loss, splenomegaly and lymphadenopathy. Lymph node biopsy and/or splenic and bone marrow biopsies are essential for pathological diagnosis and staging of the disease. For proper categorization of different subtypes of CLPD immunophenotyping, immunohistochemistry and genetic studies are mandatory **[1]**. Different staging systems are established for clinical staging of lymphoproliferative disorders including Ann-Arbor, Binnet, Rai, and Modified Rai **[1]**.

Angiogenesis is the formation of new blood vessels from the pre-existing ones, it is a critical process that occurs in the body both in health and disease. Physiologically it is important for wound healing and formation of placenta. The healthy body controls the formation of new blood vessels through the balance between positive regulators as fibroblast growth factors and vascular endothelial growth factor and negative regulators as transforming growth factor B and platelet factor **[4]**.

Endthothelial cell markers such as thrombomodulin, von Willebrand factor and VEGF play an important role in malignancy. Thrombomodulin is an integral membrane protein expressed on the surface of endothelial cells. It functions as a cofactor in thrombin induced activation of protein C in the anticoagulant pathway. As it is expressed in high density by a restricted number of cells including endothelial and mesothelial cells it is used as endothelial cell marker **[5]**.

Von Willebrand factor plays an important role in primary hemostasis by promoting platelet adhesion to the subendothelium at site of vascular injury. It is stored in alpha granules of megakaryocytes and endothelial cells (Weibel-Palade bodies). It is used as an endothelial cell marker to highlight the endothelial cells **[6]**.

Vascular endothelial growth (VEGF) factor plays an essential role in vasculogenesis during embryogenesis, physiologic angiogenesis and the neovascularization of malignancy. It is a tumour derived angiogenic factor that promotes the formation of endothelial lining of tumour vessels by recruitment of highly proliferative circulating endothelial precursors (CEPs, angioblast) from the bone marrow, haematopoetic stem cells, progenitor cells, monocytes and macrophages **[7]**.

Aim of the work:

The aim of this work is to study the role of angiogenesis in the pathogenesis of CLPD.

MATERIAL AND METHODS

This study was carried out on 26 patients with chronic lymphoproliferative neoplasms (14 male and 12 female) 12 patients had FL, 8 with DLCL, and 6 CLL. Cases were selected from the Hematology Department of the Medical Research Institute, Alexandria University over a period of 18 months. All selected cases have infiltrated bone marrow biopsies. Ten bone marrow biopsy sections for lymphoma patients showing no infiltration were also included in the study as control group.

Exclusion criteria:

- Antiangiogenic drugs.
- Other causes of angiogenesis as hepatitis C.

All patients and controls were subjected to the following:

1- Medical examination:

Detailed history taking and thorough clinical examination to assess the presence of lymph node enlargement, the presence of hepatomegaly and splenomegaly.

2-Routine investigations:

A- Imaging investigations:

Plain X-ray chest, abdominal ultrasound and CT abdomen.

B- General laboratory investigations including:

Complete blood picture **[8]**, ESR **[8]**, serum LDH **[9]**, liver functions tests **[10]** and kidney functions tests **[11]**.

C- Specific laboratory investigations:

Bone marrow aspiration and biopsy **[12]**.

Immunohistochemical staining of infiltrated bone marrow using the following monoclonal antibodies:

1- Mouse anti-human CDC141, Thrombomodulin clone (1009) **[13]**:

Applied at 1:25 dilutation for 60min at room temperature.

Positive control: Mesothelioma.

Staining pattern: Cell membrane.

2- Factor VIII related antigen/von Willbrand factor rabbit antibody **[14]**.

Applied at 1:100 dilution for 10min at room temperature.

Positive control: Tonsil.

Staining pattern: Cytoplasmic.

3- Vascular endothelial growth factor epitope specific rabbit antibody **[15]**.

Ready to use for 10min at room temperature.

Positive control: Tumor cells in hemangiosarcoma.

Staining pattern: Cytoplasmic, cell surface and extra-cellular matrix.

Principle of immunohistochemical staining:

The method consists of a labeled streptavidin biotin immunoenzymatic antigen detection system. This technique involves the squentional incubation of the specimen with an unconjugated primary antibody specific to the target antigen, a biotinylated secondary antibody which reacts with the primary antibody, enzyme-labeled streptavidin and substrate-chromogen.

Intensity of immunocytochemical staining was evaluated by taking both intensity of the color reaction and percentage of cells which exhibited the positive reaction. Final results represented the product of the two parameters to calculate the IRS.

% positive cells	Grading of positivity	Intensity of the reaction
No positive cells	0	No positive reaction 0
<10 positive cells	1	Faint color reaction 1
10-50% positive cells	2	Moderate color reaction 2
51-80% positive cells	3	Intense color reaction 3
>81% positive cells	4	

Statistical analysis:

After data entry into a specially designed sheet using Microsoft Excel, a print out of the data was thoroughly revised and data entry mistakes were corrected. Then the file was transferred into Statistical Package for Social Science (SPSS) version 17 format and data explore was carried out. Testing normality using Kolmogorov-Smirnov test proved that data is abnormally distributed, so median and inter-quartile range (IQR) were used for descriptive statistics and non-parametric (Kruskal-Wallis and Mann-Whitney) tests were used for comparison. When Kruskal-Wallis test is significant multiple inter-group comparison (pair-wise comparison) were carried out and correction of *p* value for multiple comparison (Bonferroni correction) was done. As only comparison with the control group was significant other inter-group significant which proved to be non-significant were not mentioned in the tables. Kendall's-Tau bivariate correlation was also performed. The study adopted a 0.01 level of significance (alpha error) and beta error was set to be 20%.

RESULTS

The present study was conducted on 26 patients presented to the Medical Research Institute with chronic lymphoproliferative neoplasms. Their ages ranged from 26 to 67 years with a mean of 51.5±10.3 years, they were 14 male and 12 female with a ratio of 1.16 to 1.0.

The studied patients were classified into: Twelve patients with FL (46.15%), eight patients with DLCL (30.75%) and six patients with CLL (23.10%).

The clinical presentation of the studied patients were lymphadenopathy in 25 patients (96.15%), splenomegaly in 24 (92.3%), hepatomegaly in 21 (80.67%) and B symptoms in 19 (73.10%).

Bone marrow trephine biopsy:

The patterns of bone marrow infiltration of the studied group showed the following: Focal pattern of bone marrow infiltration in 7 (26.9%), interstitial pattern in 7 (26.9%), diffuse pattern in 6 (23.10%) and mixed pattern in 6 (23.10%). As regards the type of infiltrating cells, 12 (46.15) were infiltrated by mixed small and large cells, 9 (34.6%) were infiltrated by small cells, and 5 (19.23%) were infiltrated by large cells.

Immunohistochemical staining of the bone marrow blood vessels using the anti von Willebrand factor:

When comparing the number of blood vessel in the bone marrow sections on using anti-vWF as endothelial marker between the studied cases and the control group, Table (1), a statistically significant difference was detected (p=0.000). Cases of FL, DLCL and CLL showed statistically significant difference when each group was compared with the control group. (ZMW= 3.979, p=0.000), (ZMW=3.591, p=0.000) and (ZMW=3.286, p=0.001) respectively. (Table 1).

Immunohistochemical staining of the bone marrow blood vessels using antithrombomodulin:

On using anti thrombomodulin as endothelial cell marker there was also statistically significant difference between patients and the control group (p=0.000). On comparing the different histopathological groups with the control group, cases of FL, DLCL and CLL each group showed a statistically significant difference when compared with the control group (ZMW=3.987, p=0.000), (ZMW=3.591, p=0.000) and (ZMW= 3.293, p=0.001) respectively (Table 1).

Vascular endothelial growth factor expression in the studied cases:

VEGF expression (% of positive cells) showed statistically significant difference between cases and control group. On comparing the different histopathological groups with the control group, cases of FL, DLCL and CLL showed statistically significant difference when comparing each with the control group (ZMW= 3.983, p=0.000), (ZMW=3.580, p=0.000), (ZMW=3.273, p=0.000) respectively. Regarding the IRS, it was significantly higher in cases as compared with the control group (p<0.001), also cases with FL, DLCL and CLL each group was statistically significant when compared with the control group. (Table 1).

On comparing the different histopathological groups with each other (FL, DLCL and CLL) using anti-vWF, antithrombomodulin and vascular endothelial growth factor. There was no significant statistical difference between the three studied groups.

Immunoreactive score (IRS):

In FL cases, it ranged from 2-9 with a median (IQR) of 4.0 (2), in cases of DLCL it ranged from 2-6 with a median (IQR) of 4.0 (2), while in the six cases of CLL it ranged from 2-4 with a median (IQR) of 4.0 (2) and in the control group the median was 1 (1). (Table 1).

Correlation between various immunohisotchemical stains:

A positive correlation was encountered between the number of blood vessels detected by anti-vWF, antithrombomodulin and VEGF (Figs. 1-3).

Examples of positive immunohistochemical staining with anti-vWF, antithrombomodulin and VEGF expression are presented in (Figs. 4-9).

DISCUSSION

Development of tumours is a highly complex process in which several molecular events are required for tumour cells to achieve independent growth. One of such events is the enhancement of angiogenesis **[3]**.

Angiogenesis and proangiogenic growth factors have a known role in solid neoplasia, and there is increasing evidence that they also play a role in hematolymphoid neoplasia. Increased microvessel density has been noted in a range of hematolymphoid disorders, including multiple myeloma, non Hodgkin lymphoma, acute and chronic leukemias of lymphoid and myeloid lineages and myelodysplastic disorders **[4]**.

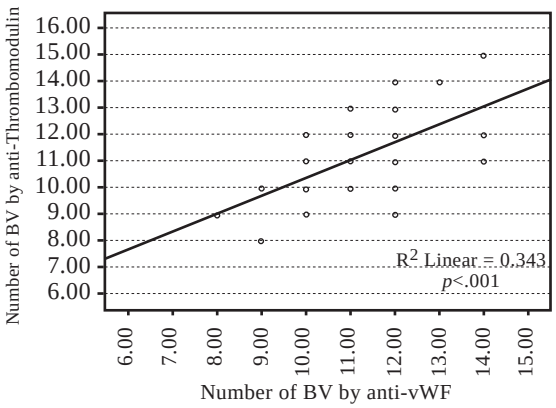

Fig. (1): Positive correlation between immunohistochemical staining of bone marrow blood vessels by anti-vWF and anti-thrombomodulin.

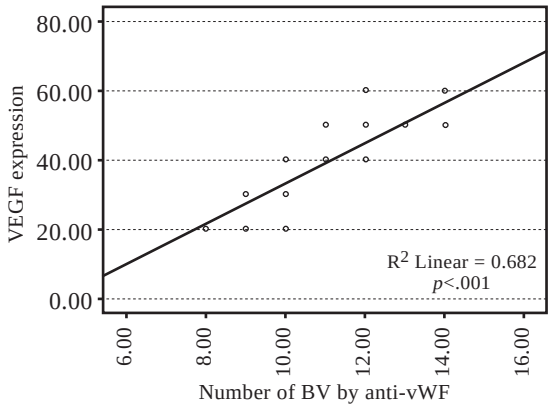

Fig. (2): Positive correlation between immunohistochemical staining of bone marrow blood vessels by anti-vWF and VEGF expression (% of positive cells).

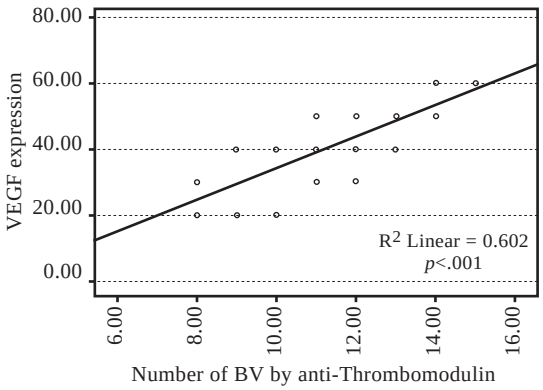

Fig. (3): Positive correlation between immunohistochemical staining of bone marrow blood vessels by anti-thrombomodulin and VEGF expression (% of positive cells).

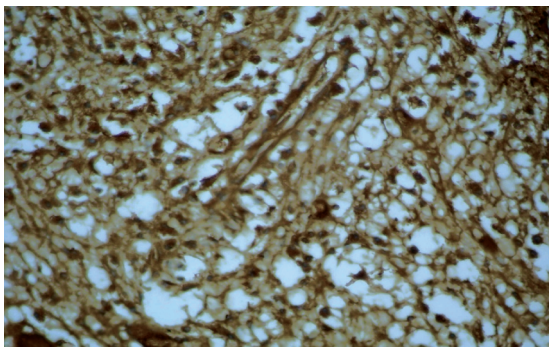

Fig. (4): Bone marrow trephine biopsy in follicular lymphoma showing positive immunohistochemical staining of bone marrow blood vessels with anti-vWF (400X).

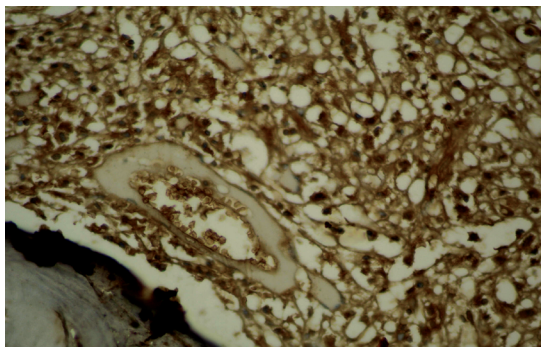

Fig. (5): Bone marrow trephine biopsy in follicular lymphoma showing positive immunohistochemical staining of bone marrow blood vessels with anti-vWF (400X).

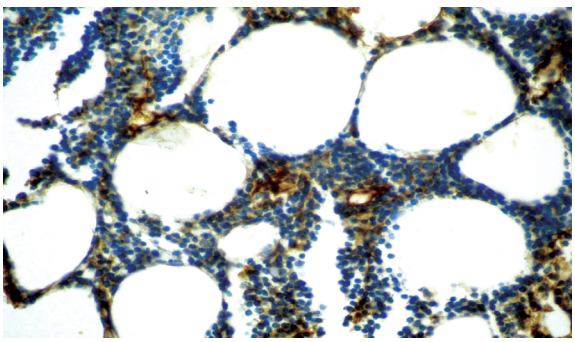

Fig. (6): Bone marrow trephine biopsy in follicular lymphoma showing positive immunohistochemical staining of bone marrow blood vessels with anti TM (400X).

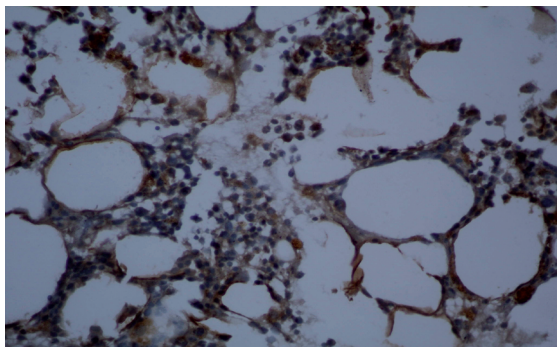

Fig. (7): Bone marrow trephine biopsy in diffuse large cell lymphoma showing positive immunohistochemical staining of bone marrow blood vessels with anti TM (400 X).

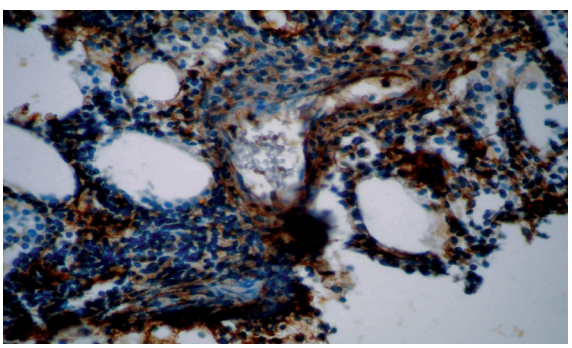

Fig. (8): Bone marrow trephine biopsy in diffuse large cell lymphoma showing positive immune histochemical staining with vascular endothelial growth factor (the positivity was 60%).

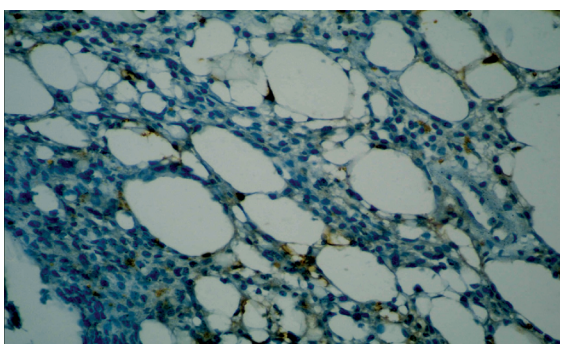

Fig. (9): Bone marrow trephine biopsy in chronic lymphocytic leukemia showing positive immunohistochemical staining with vascular endothelial growth factor (the positivity was 20%).

Table (1): Comparison of immunohistochemical staining of bone marrow BV with anti-vWF and anti-thrombomodulin and VEF expression among the studied patients with different histopathology.

	Patients with FL (n = 12)	Patients with DLCL (n = 8)	Patients with CLL (n = 6)	Control group (n = 6)	Significance
Anti-vWF:					
Median	11.5	12.0	10.0	2.50	X^2(KW)= 27.703
IQR	2.75	1.75	2.0	1.50	*p*= 0.000*
Z_{MW}	3.979	3.591	3.286		
p value	0.000*	0.000*	0.001*		
Anti-Thrombomodulin:					
Median	12.0	11.0	9.50	3.0	X^2(KW)= 26.861
IQR	2.50	2.75	3.50	2.0	*p*= 0.000*
Z_{MW}	3.987	3.591	3.293		
p value	0.000*	0.000	0.001*		
VEGF (%):					
Median	40.0	40.0	35.0	4.0	X^2(KW)= 25.826
IQR	17.5	10.0	20.0	4.0	*p*= 0.000*
Z_{MW}	3.983	3.580	3.273		
p value	0.000*	0.000*	0.001*		
IRS:					
Median	4.0	4.0	4.0	1.0	X^2(KW)= 26.216
IQR	2.0	2.0	2.0	1.0	*p*= 0.000*
Z_{MW}	4.061	3.661	3.293		
p value	0.000*	0.000*	0.001*		

IRS : Immunoreactive score.
IQR : Interquartile range.
$X^2_{(KW)}$: Chi square of Kruskal-Wallis test.
$Z_{(MW)}$: Z of Mann Whitney test (comparing each group with the control group). There is no other intergroup significance.
p : Probability of error (level of significance).
* : Significant difference (after correction for multiple comparisons).

The present study was conducted on twenty six patients with chronic lymphoproliferative neoplasms: Twelve patients with FL, eight patients with DLCL, and six patients with CLL.

Immunohistochemical staining of bone marrow blood vessels using anti von Willibrand factor and antithrombomodulin as well as histochemical studies of VEGF expression in bone marrow trephine biopsy sections were investigated in our patients as markers of angiogenesis.

In the present study we detected a significant increase in the number of bone marrow blood vessels when compared to the normal controls ($p<0.001$).

Our results are in agreement with El-Sorady et al., **[16]** who studied angiogenesis in 20 patients with NHL and 20 patients with Hodgkin disease, using immune-histochemical staining of bone marrow blood vessels by anti-vWF as endothelial cell marker in bone marrow biopsy sections. They observed that patients with hematological malignancies generally had significantly higher bone marrow microvessel counts when compared with the control group.

Foss et al., **[17]** stated that malignant lymphomas are heterogenous with respect to their microvasculature. They observed that vascularity was prominent in HD but not in most cases of low grade B cell lymphoma.

In the present study immunohistochemical staining using anti TM as endothelial cell marker in cases with FL as well as cases with DLCL showed a high statistically significant increase in number of BM blood vessels when compared with the control group.

Ribatti et al., **[18]** in their study concerning angiogenesis in NHL demonstrated that more intense vascularization has been described in B-cell NHL compared with the benign lymphadenopathies. Our results are also in line with other reports which demonstrated increased bone marrow blood vessels in bone marrow biopsy sections in different hematological malignancies, when compared to the control group. **[19,20]**.

These results are also concomitant with Gratzinger et al. (2007), **[21]** who assessed angiogenesis in cases of diffuse large B cell lymphoma, however they assessed the vascu-

larity immunohistochemically in lymph node biopsy sections. They stated that diffuse large B cell lymphoma specimens showed higher microvessel density when compared with the controls, implying that lymphoma cells induce local tumour angiogenesis. Moreover, they also stated that this microvessel densities showed a broad distribution which may offer differential access to the vascularly distributed nutrients, growth factors and chemotherapeutics.

In this study we could not observe a statistically significant difference in the number of BM blood vessels counted by using anti-vWF and those counted by using anti TM antibodies, and this could be attributed to the small sample size.

List et al., **[22]** in their study on lymph node biopsies stated that cellular expression of VEGF is common in cases with NHL. El-Sorady et al., **[16]** measured VEGF levels in the serum of their NHL patients, and they demonstrated that they significantly exceeded those of the control group. They stated that VEGF appears to be one of the most relevant angiogenic factors, since it's expression is closely related with the vessel density in most hematological malignancies. Foss et al., **[17]** found that VEGF expression in lymphomas proved to be almost entirely restricted to reactive cells, which are probably fibroblasts. They suggested that the process of angiogenesis differs in epithelial and lymphoid tumours and that the induction of angiogenesis through VEGF in malignant lymphomas is an indirect process involving reactive cells.

In this study we detected a strong positive correlation between the number of blood vessels counted by anti-vWF and thrombomodulin and the degree of VEGF expression. Cases which showed the highest number of BM blood vessels also had the highest VEGF expression.

These results are in accordance with Gartzinger et al., **[21]** although they assessed the relationship among the mirovessel density and expression of vascular endothelial growth factor in lymph node biopsy sections. They stated that diffuse large B cell lymphoma specimen showing higher local vascular endothelial growth factor expression showed correspondingly higher microvessel density. In addition, they also found that local vascular endothelial growth factor expression was higher in those specimens showing higher expression of the receptors of the growth factor, suggesting an autocrine growth promoting feedback loop.

In the present study VEGF expression did not show any statistical difference between cases of FL and cases DLCL.

Ruan et al. (2008), **[23]** reported that VEGF expression by neoplastic cells had been demonstrated in aggressive subtypes of lymphoma including peripheral T cell lymphoma, diffuse large B cell lymphoma, mantle cell lymphoma, primary effusion lymphoma and indolent histologies such as CLL/SLL, they also stated that only a minority of indolent follicular lymphoma cases showed variable expression of VEGF, and that increased VEGF expression had been associated with areas of transformation from indolent B cell lymphoma to aggressive DLBC and poor prognostic subgroups within DLBCL **[23]**.

In contrast Gratzinger et al. in another study (2008), **[24]** investigated the prognostic significance of VEGF expression, VEGF receptors and microvessel density in diffuse large B cell lymphoma treated with anthracycline-based chemotherapy, and they stated that no correlation between increased MVD and VEGF expression has been found. The investigations also concluded that increased tumour vascularity was associated with poor overall survival and was independent of the international prognostic index (IPI) score. Other studies found no correlation between baseline MVDs and VEGF expressions, IPI score or clinical outcome. These studies provided a glimpse into the heterogeneity and complexity of the angiogenic processes in DLBCL **[24]**.

Koster et al., **[25]** stated that in follicular lymphoma, it is well accepted that MVDs are significantly higher in interfollicular as opposed to intrafollicular regions. They also reported that increased vascularity pretreatment predicted favorable outcome.

Another possible explanation for Koster et al., **[25]** findings is that patients with follicular lymphoma with increased angiogenesis and which are characterized by a high MVD are more susceptible to the antiangiogenic effects of IFN-α2b therapy than those with low vascularity.

In the present study patients with CLL showed significant increase in the number of blood vessels in the bone marrow sections using anti-vWF and anti TM, compared with normal controls ($p<0.001$). Molica et al., **[26]** found significant increase of BM angiogenesis in cases of B-CLL, using anti-vWF as endothelial marker. They also stated that level of BM angiogenesis in hematologic malignancies is a complex process related to an interaction of an array of angiogenic and antiangionic factors released into the microenviroment. They also reported that the extension of microvessel area predict the risk of progression of the Binet stage, suggesting that the microvessel area should be preferred for prognostic assessment.

Kini et al., **[27]** found a significant positive correlation between the Rai clinical stage at the time of the biopsy, and microvessel density so they correlated the extent of BM involvement by B-CLL and the microvessel numbers, and reported that there was positive correlation between both. Regarding our cases, in the present study they were all in Binnet stage C, so similar correlation between the stage of the disease and microvessel count could not be done. Moreover Frater et al., **[28]** found in their study that CLL patients had higher MVD compared to controls.

The present study detected a strong positive correlation between microvessel counts obtained by anti-vWF and anti TM antibodies, two highly specific endothelail cell markers, no significant difference was found between them. Other studies have also reported that vWF, although highly specific for the vasculature, was partially absent in the capillary endothelium of tumour tissue, this explained the lower microvessel count they found in the bone marrow sections stained by this marker. They also stated that megakaryocytes are stained with anti-vWF but they were easily distinguishable by their morphology so they suggested that TM staining was a reliable tool for quantification of angiogenesis.

In contrast to these results Aguayo et al., **[29]** studied angiogenesis in cases of chronic leukemias and they used anti-vWF to highlight endothelial cells, they stated that they found no increase in BM neovascularisation in cases of CLL despite increase in cellularity. When they compared BM cellularity with vascularity, there was no correlation and vascularity appeared independent of cellularity. They suggested that other factors in bone marrow stroma or leukemic process may be important in determining the level of vascularity in these diseases.

As regards VEGF expression in CLL patients, there was statistically significant increase in the percentage of positive cells, intensity of the reaction and immunoreactive score.

Chen et al., **[30]** studied angiogenesis in CLL cases, they stated that VEGF expression is increased up to 7 folds. VEGF produced by B-CLL cells stimulates endothelial cell proliferation and angiogenesis.

In another report by Kay et al., **[31]** they demonstrated increase in VEGF and basic fibroblast growth factor (bFGF) in the culture supernatant of CLL cells grown in vitro and upregulation of mRNA encoding VEGF and its receptors as well as bFGF, suggesting that angiogenic factors are important in the biology of the malignant B-cell clone **[30]**.

In conclusion, the increase in bone marrow blood vessels, which is measured by immunohistochemical staining with anti-vWF and anti-thrombomodulin and the increased expression of VEGF suggests that increased angiogenesis may play a role in the pathogenesis of the disease.

Serial immunohistochemical staining of bone marrow microvessel densities in the follow-up of cases is recommended that might be of prognostic value. Evaluation of angiogenesis after treatment is also recommended to judge patient's response to therapy and to help the decision of adding anti-angiogenic drugs to protocols of treatment.

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