Expression of VEGF-A and HIF1 alpha in diffuse large B-cell lymphoma and low grade follicular lymphoma

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This study has been carried out at the Institute of Pathology, Faculty of Medicine, Cyril&Methodius University of Skopje, and Institute of Pathology, University Clinical Centre of Kosova

Mentors: Prof. Gordana Petrusevska, MD, PhD
Prof. Slavko Gasparov, MD, PhD

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In memory of my sister Lavdije.

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LIST OF ABBREVIATIONS

DLBCL	Diffuse Large B Cell Lymphoma
FL	Follicular Lymphoma
NHL	Non-Hodgkin's Lymphomas
WHO	World Health Organization
GEP	Gene expression profiling
R-CHOP	is the acronym for chemotherapy treatment. Rituximab, Cyclophosphamide, Doxorubicin, Vincristine, Prednisone
SPARC	Secreted protein, acidic and rich in cysteine
CTGF	Connective tissue growth factor
VEGF-A	Vascular Endothelial Growth Factor – A
VEGFR	Vascular Endothelial Growth Factor Receptor
HIF-1α	Hypoxia Inducible Factor - 1α
PDGF	Platelet Derived Growth Factor
PDGFR	Platelet Derived Growth Factor Receptor
IPI	International Prognostic Index
FLIPI	Follicular Lymphoma International Prognostic Index
OS	Overall survival
LDH	Lactate Dehydrogenase
ECOG	Eastern Cooperative Oncology Group
R-IPI	Revised International Prognostic Index
VPF	Vascular Permeability Factor
COX2	Cyclooxygenase 2
bFGF	Basic Fibroblast Growth Factor
PIGF	Placental Growth Factor
SDF1	Stromal cell-derived Factor 1
MMP9	Matrix Metallopeptidase 9
BMC	Bone Marrow-derived Cells

MVD	Microvessel Density
vWF	von Willebrand factor
O_2	Oxygen
Ang	Angiopoietin
LOX	Lysyl Oxidase
CAIX	Carbonic Anhydrase IX
CXCR	Chemokine Receptor
EMT	Epithelial-to-Mesenchymal Transition
PDH	Prolyl-hydroxylase
VHL	von Hippel-Lindau
Ub	Ubiquitination
HRE	Hypoxia Response Elements
GLUT -1	Glucose Transporter 1
IGF 2	Insulin-like Growth Factor 2
DLK-1	Delta-like 1
COL5A1	Collagen, type V, α1
ABCB1	ATP-binding cassette transporter B1
MCP-1	Monocyte Chemoattractant Protein–1
ADM	Adrenomedullim
DLL	Delta-like ligand
Flt-1	fms-related tyrosine kinase 1
KDR	Kinase Insert Domain Containing Receptor
NOS	Nitric Oxide Synthases
PAI-1	Plasminogen Activator Inhibitor–1
SCF	Stem Cell Factor
Tie-2	TEK tyrosine kinase endothelial
TIMP	Tissue Inhibitor of Metalloproteinases
DAB	3,39-diaminobenzidine tetrahydrochloride
IHS	Immunohistochemical Score
PFS	Progression Free-Survival
PCLBCL-LT	Primary cutaneous B-cell lymphoma-leg type

CVP	Cyclophosphamide, vincristine, and prednisone
IFN	Interferone
CLL	Chronic Lymphocytic Leukemia
MDS	Myelodysplastic Syndrome

1. INTRODUCTION AND BACKGROUND OF THE DISSERTATION

1.1. Diffuse large B-cell lymphoma

Diffuse large B-cell lymphoma NOS (DLBCL) is a neoplasm of large B lymphoid cells with nuclear size equal to or exceeding normal macrophage nuclei or the size more than twice size of a normal lymphocyte that has a diffuse growth pattern (1). DLBCL displays striking heterogeneity at the clinical, genetic, and molecular levels (2). Diffuse large B-cell lymphoma is the most common lymphoid malignancy in adults. At present time, in Europe and USA the annual incidence of Non-Hodgkin's Lymphomas (NHL) is estimated to be at 15–20 cases/100,000. Based on the data of the International NHL study group (Non-Hodgkin's Lymphoma classification Project 1997) and the fourth edition of the WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues, DLBCL accounts for approximately 31% of all NHL in Western Countries and 37% of B-cell tumors worldwide. The median age of DLBCL falls between the sixth and seventh decade, although other types of aggressive NHL present at a lower median age (3). This category was included both in the REAL and WHO Classification aiming to describe all malignant lymphomas characterized by the large size of the neoplastic cells, B-cell derivation, aggressive clinical presentation, and the need for highly effective chemotherapy regimens (4). Morphological, biological and clinical studies have subdivided diffuse large B-cell lymphomas into morphological variants, molecular and immunophenotypical subgroups and distinct disease entities. However, a large number of cases remain that may be biologically heterogeneous, but for which there are no clear and accepted criteria for subdivision. These are classified as DLBCL, not otherwise specified (NOS) (1).

In further support to the concept that signaling from the tumor microenvironment contributes to the progression of DLBCL and ultimately patient outcome, a GEP study with R-CHOP-treated patients demonstrated two "stromal" signatures: "stromal-1" and "stromal-2". The stromal-1 signature was associated with increased patient survival and included expression of genes related to the extracellular matrix and histiocytes, such as secreted protein, acidic and rich in cysteine (SPARC) and connective tissue growth factor (CTGF). The stromal-2 signature includes endothelial and angiogenesis-related

genes and is targetable with anti-vascular endothelial growth factor (VEGF) based drugs, platelet derived growth factor receptor (PDGFR), angiopoietin/tyrosine kinase with immunoglobulin-like and EGF-like domains-2(ANG/TIE2) activity, and inhibitory effects on the CXCR4-CXCL12 axis (5).

Diffuse Large B-Cell Lymphoma (DLBCL) is a curable lymphoma. Although CHOP chemotherapy with rituximab remains a standard therapeutic approach for most patients with DLBCL, we anticipate that novel agents will be included in treatment regimens for many patients in the near future (6).

On immunohistochemistry DLBCL is positive for CD19, CD20, CD22, CD45, CD79a, PAX5. Additional useful markers are Bcl-2, p53 and Ki-67 (1, 3).

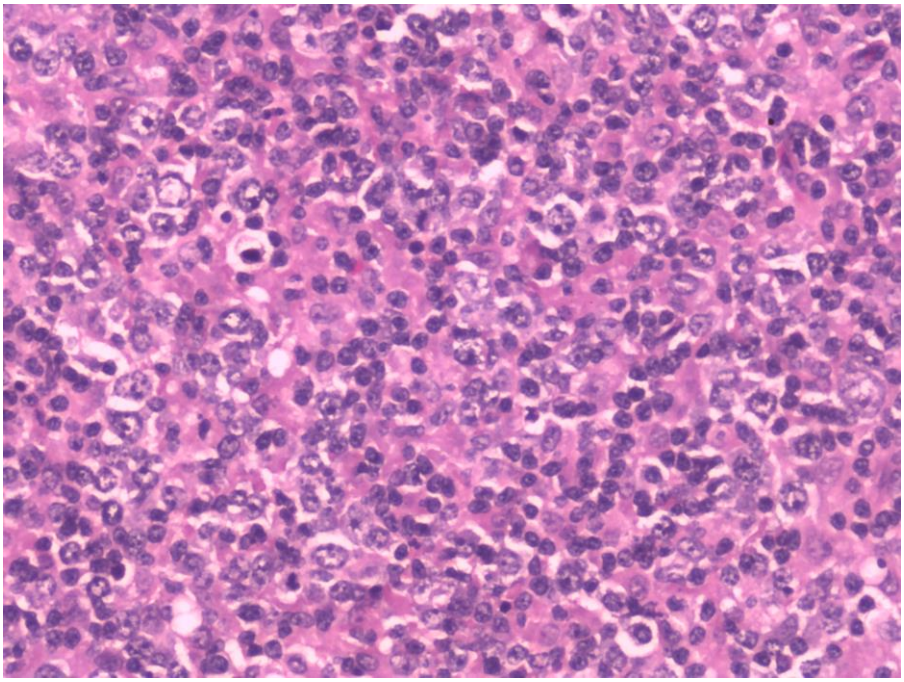

Figure 1. Histology of Diffuse Large B-cell lymphoma (HE 200X)

1.1.1. International Prognostic Index (IPI)

The International Prognostic Index (IPI) is the widely accepted prognostic factor index for patients with aggressive lymphomas. It was introduced by Shipp et al., in the 1990s and was based on an individual case-based prognostic factor analysis of cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) –like regimens with overall survival (OS) as end point.

The IPI includes five factors: age (≤60 years *v* >60 years), lactate dehydrogenase (LDH) value (≤upper limit of normal [ULN] *v* >ULN), performance status (Eastern Cooperative Oncology Group [ECOG] 0,1 *v* >1), Ann Arbor stage (I/II *v* III/IV), and the number of extranodal sites involved (0,1 *v* >1). The age-adjusted IPI (aaIPI) for younger patients includes the factors LDH, performance status, and stage. The IPI score separates four prognostic groups based on the number of factors present: (0, 1: low risk group; 2: low-intermediate-risk group; 3: high intermediate- risk group; and 4, 5: high-risk group). The IPI has been widely used and found effective and reproducible when various conventional, high-dose, and dose-dense regimens were analyzed (7).

R-IPI identifies 3 distinct prognostic groups with significantly different outcomes. Patients with zero risk factors fall into a “very good” prognostic group with more than a 90% chance of long-term progression-free survival. Patients with 1 or 2 risk factors fall into a “good” prognostic group with an approximately 80% chance of long-term progression-free survival. Finally, patients with 3, 4, or 5 risk factors fall into a “poor” risk group with a long-term chance of cure in the range of 50% (8).

Combination chemotherapy has transformed aggressive non-Hodgkin’s lymphoma from a fatal disease into one that is often curable. However, many patients still die of their disease, underscoring the need for more accurate methods of prospectively identifying patients with different long-term prognoses (9).

Ziepert et al., strongly recommended staying with the IPI and not changing to other scoring systems or introducing novel risk factors as long as there is no convincing evidence that new factors or new scoring systems show distinct advantages over the well-established IPI (10).

1.2. Follicular lymphoma

Follicular lymphoma (FL) is a neoplasm composed of follicle centre (germinal centre) B-cells (typically both centrocytes and centroblasts/large transformed cells), which usually has at least a partially follicular growth pattern (11). FL is a prototype of low grade lymphoma (12). FL accounts for about 20% of all lymphomas with approximately 30,000 patients newly diagnosed per year, and it affects predominantly adults, with median age in the 6th decade, male: female ratio is 1:1,7 (11, 12). FL predominantly involves lymph nodes, but also spleen, bone marrow, peripheral blood and Waldeyer ring (11). FL is graded according to the proportion of large cells (centroblasts) in ten neoplastic follicles, expressed per 40x high-power microscopi field (hpf). Grade 1 and grade 2 (low grade) have a marked predominance of centrocytes (grade 1=0-5 centroblasts/hpf; grade 2 = 6-15 centroblasts/hpf), and grade 3 (high grade) have more than 15 centroblasts/hpf (11, 12, 13). Grade 1 and 2 were combined in the most recent iteration of the WHO classification due to poor interobserver reproducibility and the general consensus that more detailed distinction between FL grade 1 and 2 was clinically insignificant. FL grade 3 is divided in 3A, which retains centrocytes, and 3B, which consists of follicles composed of centroblasts (11, 12). The majority of follicular lymphomas are grade 1-2 (80-90%) (11).

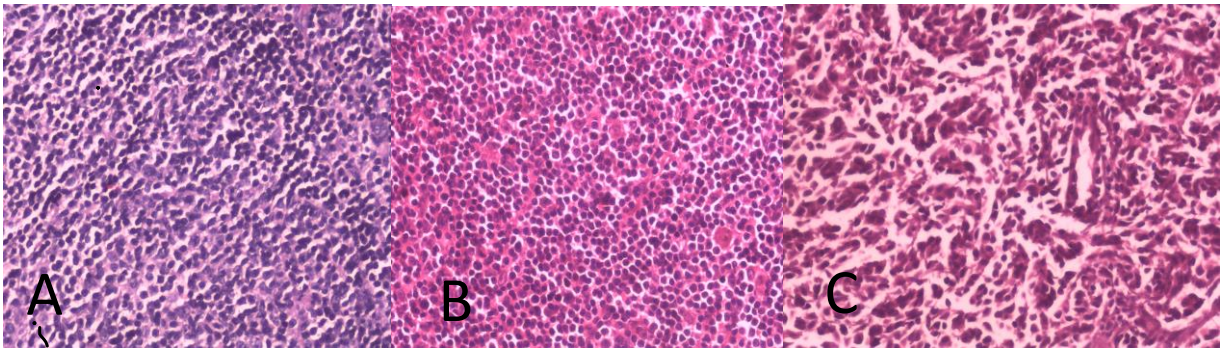

Figure 2. Follicular lymphoma grading (A) grade 1, (B) grade 2, and (C) grade 3 (200X).

The tumor cells usually express CD19, CD20, CD22, CD79a, Bcl-2, Bcl-6, CD10, and are negative for CD5 and CD43. Some cases especially grade 3B may lack Bcl-2 (11).

1.2.1. Follicular Lymphoma International Prognostic Index

As a prognostic model for FL, the Follicular Lymphoma International Prognostic Index (FLIPI) was first developed in a multinational retrospective study. Factors included in FLIPI are age >60 years, Ann Arbor stage III or IV, hemoglobin less than 12 g/dL, elevated levels of serum lactate dehydrogenase (LDH), and ≥5 involved nodal sites (14). The Follicular Lymphoma International Prognostic Index (FLIPI) is the result of a large international cooperative effort in which clinical data were collected for 4167 patients with FL diagnosed between 1985 and 1992. From this database, a prognostic index with five adverse factors was derived and validated. The index is able to separate 3 risk groups of approximately equal size with clear differentiation of long-term prognosis. Prognosis is closely related to the extent of disease at diagnosis. The FLIPI is strong predictors of outcome (8, 14, 15). FLIPI was developed in the pre-rituximab era. A newer prognostic model, named FLIPI2, was proposed through the analysis of prospectively collected data from patients who were treated with rituximab-containing chemotherapy (16). In the FLIPI2, new parameters were added, including elevated serum β2-microglobulin, largest involved node longer than 6 cm in diameter, and bone marrow (BM) invasion, hemoglobin, and age (16). Although FLIPI is still widely used, a recent Italian study reported the validation of FLIPI2 and showed that the prognostic value of FLIPI2 was slightly better than FLIPI (17, 18).

Follicular Lymphoma International Prognostic Index (FLIPI)

	Score	
Factor	**0**	**1**
Age	≤60 yr	>60 yr
Ann Arbor stage	I/II	III/IV
Hemoglobin level	≥12g/dl	<12g/dl
Number of nodal areas	≤ 4	> 4
Serum LDH	≤ Normal	> Normal

Risk group	Number of Adverse Factors
Low-risk	0-1
Intermediate risk	2-3
High risk	4-5

Figure 3. Follicular Lymphoma Prognostic Index (FLIPI). Modified from Ganti AK et al. (19).

1.3. Angiogenesis

Tumors require nutrients and oxygen to grow. The formation of new tumor-feeding blood vessels from preexisting vasculature, named angiogenesis, provides these substrates. This is critical for the development of human tumors and also a prerequisite for metastasis (20). Angiogenesis means the process of creation or formation of new blood vessels, a critical natural process that occurs in the body both in health and in disease, and exerts a crucial role in the development of various tumors (21, 22, 23). Angiogenesis has been focus of much research. Since 1970s, Folkman hypothesized, that angiogenesis is required for tumor growth. While, at one time, it was expected that manipulating angiogenesis might cure or at least control most cancers, nowadays we realize that there is much to investigate and learn in this field in order to achieve this goal (24, 25, 26, 27, 28).

In evaluation of angiogenic changes it is very important to understand the neovascularisation process. This process can be divided in four phases:

- Degradation of extracellular matrix;

- Migration of endothelial cells;
- Cell proliferation, and
- Structural reorganization (26, 29, 30).

Tumor angiogenesis is influenced by a wide variety of regulatory and growth factors. Proangiogenic factors secreted by tumor cells and/or host factors stimulate endothelial cells to proliferate and to form new blood vessels that are qualitatively poor and often leaky (31). Neoplastic growth and progression in solid and hematological malignancies is associated with the formation of new blood vessels (32, 33, 34, 35, 36, 37, 38, 39).

In recent years, much has been learned about the stimulators and inhibitors of angiogenesis, and members of the VEGF family have emerged as prime mediators of this process (40, 41, 42). The VEGF/VEGFR pathway is a key mediator of angiogenesis and VEGF-A acts as a potent tumor angiogenic factor. VEGF-A stimulates the growth of new blood vessels, which provide tumors with needed oxygen and nutrients. Expression of VEGF-A has been shown to be regulated at the transcriptional and translational levels (43). The original peptide growth factor vascular endothelial growth factor (VEGF), first described as vascular permeability factor (VPF) and now denoted VEGF-A, was identified in the 1980s (38).

Vascular endothelial growth factor A (VEGF-A) is one of the most important mediators of angiogenesis, and VEGF-A expression is stimulated by intratumoral hypoxia, which, in turn, depends on the proliferative activity of the tumour. VEGF-A binds to its receptors Flk-1 and Flt-1 with tyrosine kinase activity to induce endothelial cell proliferation (Flk-1) and further capillary tube formation and monocyte migration (Flt-1) (33). The inducible enzyme cyclooxygenase 2 (COX2) is an additional important mediator of both angiogenesis and tumour growth (44), and one of the downstream actions of its prostaglandin substrates is VEGF-A production and release (45).

The neoangiogenic process in cancer is critically influenced by the local tumor microenvironment (46, 22).

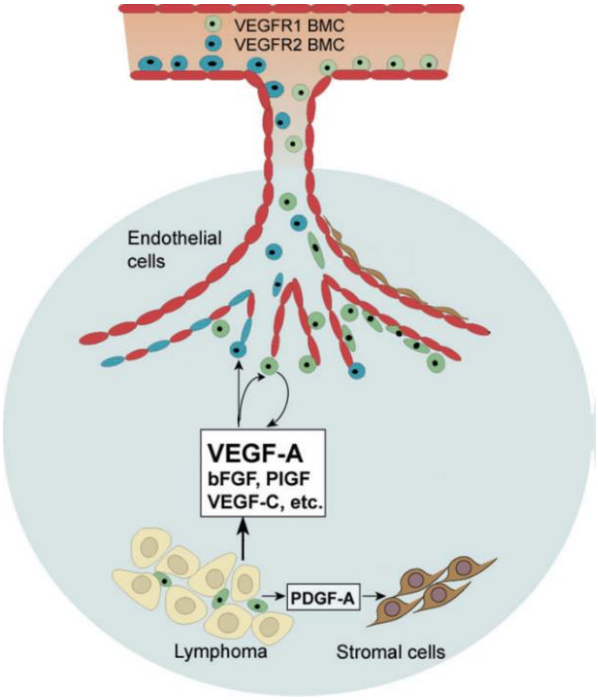

Figure 4. Overview of the lymphoma vascular microangiogenesis. Tumor cells produce VEGF-A and other angiogenic factors such as bFGF, PlGF and VEGF-C which promote neoangiogenesis via at least two mechanisms: sprouting angiogenesis of mature resident endothelial cells and vasculogenesis from recruitment of bone marrow-derived progenitor cells. (B) VEGF-A also supports the survival, proliferation and migration of lymphoma cells which express VEGFR1 and VEGFR2 in an autocrine fashion. (C) Malignant stroma, composed of fibroblasts, inflammatory and immune cells, provides additional angiogenic factors. Tumor-associated fibroblasts produce chemokines such as SDF-1, which recruits bone marrow-derived angiogenic cells. Tumor-associated macrophages produce VEGF-A, VEGF-C, and MMP-9, among others, to support endothelial proliferation. Tumor cells may also release stromal cell-recruitment factors, such as PDGF-A. Modified from Ruan J et al., (22).

The importance of angiogenesis and VEGF in solid tumors is well known. It has been shown that VEGF has a prognostic significance in many types of solid tumors. In some solid tumors, it has been shown that angiogenic activity correlates with metastatic potential and with unfavourable prognosis, for example in non-small-cell lung cancer

(47), in invasive prostate cancer (48), gastric carcinoma (49), breast cancer (50, 51, 52), etc. Surprisingly, very few studies have addressed the role of angiogenesis in the growth of human lymphomas, although several reports have implicated up-regulation of VEGF and VEGF receptors (VEGFR) in mediating lymphomagenesis (53, 54). Vascular endothelial growth factor-A mediated signaling has at least two potential roles in diffuse large B cell lymphoma: potentiation of angiogenesis, and potentiation of lymphoma cell proliferation and/or survival induced by autocrine vascular endothelial growth factor receptor-mediated signaling (55).

Vascular endothelial growth factor-A (VEGF-A)-mediated angiogenesis has received considerable attention in the context of solid neoplasia, particularly with the clinical use of anti-VEGF-A antibodies and small molecule VEGF receptor (VEGFR) inhibitors. More recently the concept of tumor vascularity has been applied to hematolymphoid neoplasia, with studies quantitating micro-vessel density in a variety of lymphomas (37, 55). In addition to its role in tumor angiogenesis, however, VEGF-A has an additional potential role in the context of hematolymphoid malignancies: that of an autocrine growth factor, acting on lymphoma cells directly through VEGF receptors (38).

Given the rapidly increasing availability of a variety of pharmaceuticals targeted at the VEGF-A pathway, the role of angiogenesis and VEGF-A signaling in diffuse large B-cell lymphoma is of significant interest (55).

In non-Hodgkin's lymphomas, expression of angiogenic factors in cell lines (35) and neoplastic tissue (54, 56, 57) has been demonstrated. Although these studies suggest a role for angiogenesis in lymphomas several questions remain unanswered. First, it is not clear whether high microvessel density (MVD) is associated with more aggressive lymphomas. A group of authors (57, 58) reported that MVD is higher in lymphomas than in reactive lymph nodes as well as in aggressive than in indolent lymphomas. However, others have found MVD in reactive lymph nodes to be higher (59) or comparable (60) to that observed in lymphomas, including large cell lymphomas (61).

Several studies have shown that serum angiogenic factor elevations (e.g., VEGF-A, endostatin), VEGF-A expression and increased microvessel density (MVD) are

predictive of poor prognosis and associated with higher tumour grade or high-grade transformation in non-Hodgkin's lymphomas. Other groups published different results (32, 62).

Although the related angiogenic factors are intensively investigated in various tumors, determination of microvessel density (MVD), a measure of the degree of angiogenesis, is one of the most frequently used parameters for quantifying angiogenesis in cancers (63).

In 1991, Weidner et al., (64) first reported a prognostic significance of tumor angiogenesis in patients with breast cancer.

Microvessel density (MVD) is a surrogate marker which particularly reflects tumor angiogenesis and has been examined as a potential prognostic marker in numerous tumors (65). Recent studies have shown enhanced angiogenesis in lymphomas, both Hodgkin's and non-Hodgkin's (66). The results in NHLs have been conflicting in that few studies have demonstrated higher MVD in aggressive subtypes of NHL (57, 67), while others have shown higher MVD in indolent lymphomas (68). Some authors have also explored association of MVD with angiogenic factors and receptor expression (69). In some studies, high MVD, as a variable of increased tumor vascularization, was associated with a significantly more favorable outcome in terms of both progression-free and overall survival (70).

Evaluation of angiogenesis can be made by several methods, including determination of microvessel density (MVD), microvessel area, angiogenic molecular quantification within tumor, presence of angiogenic receptors within the tumor, measurement of angiogenic factors in the serum or urine of patients with cancer (51, 21, 63). Tumor neovascularization was quantified by immunohistochemistry using endothelial markers to stain microvessels, which are not seen in a conventional histologic examination. After immunostaining, the entire tumor section was scanned at low power (×40) to identify "hot spots", which are the areas of highest neovascularization. Individual microvessels were then counted under high power (×200) to obtain a vessel count in a defined area, and the average vessel count in 5 "hot spots" was taken as the microvessel density (MVD). Endothelial markers commonly used for assessing MVD include CD31, CD34 and von Willebrand factor (vWF) (34).

The positive association between MVD and patient outcome is in the contrast with the notion that, in hematologic malignancies as well as in solid tumors, an increase of angiogenesis-associated variables is related to adverse prognosis (71, 34). A report on ovarian cancer in which high intratumoral MVD is an independent predictor of complete response to paclitaxel/platinum – based chemotherapy is associated with improved progression-free survival and overall survival (72). However, this has not been confirmed by several other studies (73, 74, 75). In bladder carcinoma, a positive relationship between MVD and prognosis has been described as well, but here increased tumor vascularity was associated with inflammation in the tumor and was not an independent predictor of outcome (76).

1.4. Hypoxia

Hypoxia is a condition that happens when body or an organ is deprived of adequate oxygen supply at the tissue level. Tissue hypoxia results from the inadequate supply of oxygen (O_2) that compromises biologic functions. Hypoxia can be caused by a number of factors, such as:

- Low O_2 partial pressure (O_2) tension) in arterial blood due to, e.g., pulmonary disease or high altitude (hypoxemic hypoxia);
- Reduced ability of blood to carry O_2 as a result of anemia, methemoglobin formation, or carbon monoxide poisoning (anemic hypoxia);
- Reduced tissue perfusion, generalized or local (circulatory or ischemic hypoxia);
- Deterioration of the diffusion geometry, e.g., increased diffusion distances, concurrent versus countercurrent blood flow within microvessels (diffusional hypoxia); or
- Inability of cells to use O_2 because of intoxication, as in cyanide poisoning (histotoxic or cytotoxic hypoxia) (77).

Cells exposed to hypoxic conditions respond by reducing their overall protein synthesis by approximately 50% (78). Abundant evidence suggests that hypoxia can slow down or even completely inhibit (tumor) cell proliferation in vitro (77, 79).

Human solid tumors are invariably less well-oxygenated than the normal tissues from which they arose. This so-called tumor hypoxia leads to resistance to radiotherapy and

anticancer chemotherapy as well as predisposing for increased tumor metastases (80). Tumor microenvironment is a complex and highly dynamic environment, providing very important clues to tumor development and progression (81, 82, 83).
Direct evidence of hypoxia in human cancers has been shown most convincingly by the pioneering work of Vaupel et al., who studied the tumor oxygen supply using oxygen electrodes (77, 84). They showed that low oxygen tension in tumors was associated with increased metastasis and poor survival in patients suffering from squamous tumors of the head and neck, cervical or breast cancers (84). Severely hypoxic regions in tumors result from a combination of rapid cell division and aberrant angiogenesis (86). Hypoxia has negative implications for clinical outcome. This is probably based on two different principles: hypoxic cells are more resistant to radiotherapy and chemotherapy, and they give rise to genetic instability and more aggressive phenotypes (87).

Severe hypoxia in the presence of energy stimulates cells to apoptosis, while levels of oxygen above 0.5% prevent cell death (88). Thus, a tight regulation of cellular response in such a microenvironment is indispensable. Some cells can adapt to this change of microenvironment, avoiding necrosis and apoptosis, and survive. These cells resistant to hypoxia are believed to have more aggressive phenotypes (85). During the process of hypoxia-driven malignant progression, tumors may develop an increased potential for local invasive growth (89, 90), perifocal tumor cell spreading (84, 91), and regional and distant tumor cell metastasis (92, 93). Likewise, an intrinsic resistance to radiation and other cancer treatments may be enhanced, resulting in poor prognosis.

Hence, hypoxia is attracting particular attention in the field of tumor immune biology since hypoxic stress impacts angiogenesis, tumor progression and immune tolerance. Modulating the stromal hypoxia may in fact constitute a very potent strategy for targeted therapeutic approaches (82).

The key of regulation to hypoxia response is Hypoxia inducible factor 1α (HIF-1α). HIF-1α can induce apoptosis (94), prevent cell death or stimulate cell proliferation (95). HIF-1α is involved in embryonic development (96), tumor growth, metastasis (97), and apoptosis (98).

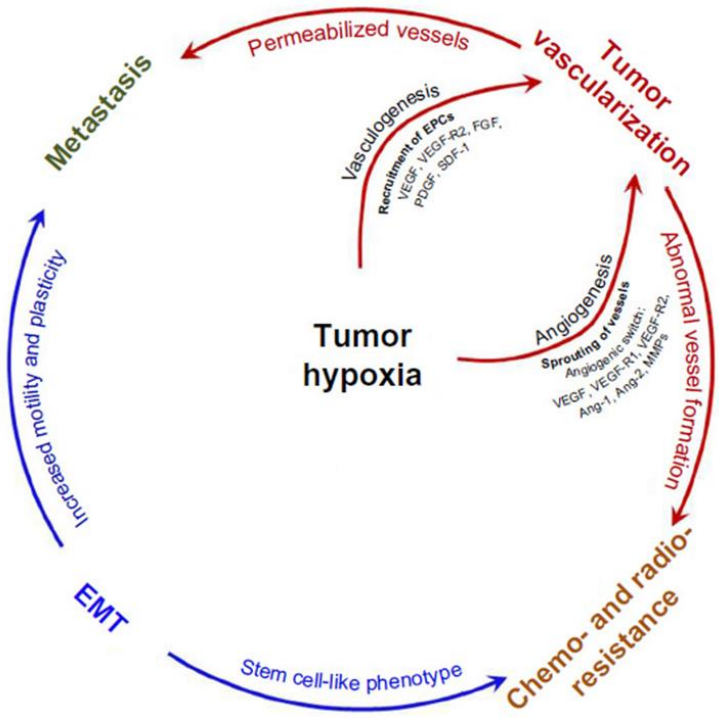

Figure 5. Hypoxia as a driving force of tumor progression and metastasis. **Notes:** Hypoxia stimulates tumor i) vasculogenesis through endothelial progenitor cells' mobilization from the bone marrow to the tumor site by VEGF, VEGF-R2, fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and stromal-derived growth factor-1 (SDF-1) and ii) angiogenesis by sprouting of the pre-existing vessels caused by increased production of VEGF, VEGF-R1, VEGF-R2, Ang-1, Ang-2, and MMPs. New blood vessels facilitate cancer cells leaving the primary tumor site, which is enhanced by increased expression of lysyl oxidase (LOX), carbonic anhydrase IX (CAIX), MMPs, integrins, and CXCR4. Hypoxic cancer cells also undergo epithelial-to-mesenchymal transition (EMT) acquiring plastic and mobile phenotype by increasing transcription factors such as Slug, Snail, and Twist and decreasing expression of adhesion molecules such as β-catenin and E-cadherin (E-cad). Chemo- and radio-resistance of patients is caused by EMT-related stemness of cancer cells and hypoxia-induced cell cycle arrest in G1 phase. Hindered drug diffusion due to anomalous vascularity is another mechanism of chemoresistance. Modified from Muz B et al (99).

1.4.1 Hypoxia inducible factor 1α (HIF-1α)

Hypoxia-inducible factor-1 (HIF-1) is a major regulator of cell adaptation to hypoxic stress and plays a critical role in tumorigenesis and angiogenesis (100). HIF-1 is a heterodimer composed of two subunits: HIF-1α and HIF-1β. Hypoxia-inducible factor α (HIF-1α) is one of the most important transcription factors and a regulator of gene products during hypoxia (101) and represents the oxygen-regulated subunit that determines HIF-1 activity. Under hypoxic conditions, HIF-1 transcriptional activity increases rapidly due to HIF-1α protein over-expression (102). Reduced oxygen diffusion within growing tumors activates angiogenesis. Hypoxia triggers the angiogenic program in a tumor microenvironment to nurture new blood vessels, which transport oxygen and dispose waste. This mechanism is transcriptionally mediated by the hypoxia-inducible factors (HIFs), HIF-1 and HIF-2. As the master regulator of vascular endothelial growth factor A (VEGF-A) during hypoxia, HIF-1 α is well studied (103).

HIF-1 promotes cancer cell growth and survival and HIF gene products protect cancer cells from chemotherapeutic agents. Constitutive expression of HIF-1α has been reported in several solid tumors (104) as well as in hematologic malignancies (105, 106), and elevated HIF levels have been linked to poor prognosis (104, 107).

HIF activity is controlled primarily through the stabilization of HIF1α and HIF2α protein subunits, which increases as cells become more hypoxic. HIFα subunits are modified by hydroxylation of two proline residues by HIF-specific prolyl-hydroxylases (PDHs) in the presence of oxygen, which leads to normoxic proteasomal degradation that is in part mediated by the von Hippel-Lindau (VHL) tumor suppressor protein (108, 109).

HIF-1a has been implicated in cancer progression and its expression correlates with tumor vascularity and aggressive behavior in a broad array of solid and hematological malignancies, i.e. acute leukemia, multiple myeloma, and Hodgkin lymphoma. The status of HIF-1a expression and its functional significance has not been well established in NHL, although the involvement of HIF appears to depend on the lymphoma histotype as well as on its specific treatment (110). Around half of follicular lymphoma (FL) patients express HIF-1α at diagnosis with a trend of worse outcome in patients with a high positive score for both HIF-1α and the HIF-2α (also termed EPAS1) protein (105).

As for MVD, no information is available on HIF-1α expression at the time of relapse/progression in NHL.

The HIF pathway regulates a host of pro-angiogenic genes (Table 1), including vascular endothelial growth factor *(*VEGF*),* angiopoietin-1, angiopoietin-2, Tie2, platelet-derived growth factor *(*PDGF*),* basic fibroblast growth factor *(*bFGF), and monocyte chemoattractant protein–1 *(*MCP-1*)* (111).

Most common pro-angiogenic factors are: VEGF, Flt-1 (VEGF-R1), Kdr (VEGF-R2), ADM, FGF, PLGF, PDGF-B, SCF, osteopontin, PAI-1, MMP, TIMP, NOS, COX-2, endoglin, α1B-adrenergic receptor, endothelin-1, semphorin 4D, integrins, leptin, endostatin, adenosin A2A receptor, oxygen-regulated protein-150, SDF-1, interleukins (IL-1, IL-2, IL-4, IL-6, IL-8, IL-10), and anti-angiogenic factors are: DLL 1-4, thrombospondin, carbonic anhydrase-9 (CA-), regulated of G-protein signaling 5, angiostatin, endostatin, canstatin and interferons (IFN-α, IFN-β, IFN-γ) (110).

Hypoxia is the principal regulator of VEGF expression, as it is a direct transcriptional target of both HIF-1α and HIF-2α (112).

1.5. Link between hypoxia and the regulation of angiogenesis

Maintenance of oxygen homeostasis is critical for the survival of multicellular organs. As a result, both invertebrates and vertebrates have developed highly specialized mechanisms to sense changes in oxygen levels and to mount adequate cellular and systemic responses to these changes. Angiogenesis is essential for normal development and neoplastic disease as tumors must develop mechanisms to stimulate vascularization to meet increasing metabolic demands. The link between hypoxia and the regulation of angiogenesis is an area of intense research and the molecular details of this connection are still being elaborated (103).

The pathological events, then lead to the restoration of oxygen homeostasis by the activation of repair mechanisms such as angiogenesis, which is the process of developing new microvessels from pre-existing ones. While post-ischemic tissue revascularization is crucial in neuronal tissues following stroke or in the heart following myocardial infarction, the activation of angiogenesis is harmful in disorders such as

macular degeneration and glaucoma, and in many types of cancer. Therefore, there is great interest in using angiogenesis regulation as a possible therapeutic method (113).

The key regulator of hypoxia-induced angiogenesis is the transcription factor hypoxia inducible factor (HIF)-1. Multiple HIF-1 target genes have been shown to modulate angiogenesis by promoting the mitogenic and migratory activities of endothelial cells (114). The key regulator of hypoxia-induced angiogenesis is the transcription factor hypoxia inducible factor (HIF)-1. Multiple HIF-1 target genes have been shown to modulate angiogenesis by promoting the mitogenic and migratory activities of endothelial cells (115).

Due to the diversified character of tumors including hypoxic and inflammatory phenotype, signaling pathways are activated simultaneously and they frequently share a number of target genes. HIF-1α and NFκB together regulate over 1,000 genes, and thus control malignant and metastatic phenotype of cancer cells since they both: i) enhance cell survival via a number of growth factors and inhibition of pro-apoptotic pathways, ii) contribute to tumor neovascularization via VEGF, VEGF receptors, COX-2, iNOS, iii) regulate cell detachment via downregulation of adhesion molecules such as cadherins, and iv) induce cell migration and invasion through matrix degrading enzymes (99).

2. HYPOTHESIS

The general hypothesis of the study is that DLBCL is characterized by higher expression of VEGF-A, HIF-1α and higher MVD than low grade follicular lymphoma which would support their role in the development of more aggressive lymphoma types.

3. AIMS

General aim of the study is to evaluate the link between hypoxia and the regulation of angiogenesis in diffuse large B-cell lymphoma and low grade follicular lymphoma.

Specific aims are:

1. Determine VEGF-A and HIF1α expression and MVD in diffuse large B-cell lymphoma (DLBCL)

2. Determine VEGF-A and HIF1α expression and MVD in low grade follicular lymphoma

3. Determine VEGF-A and HIF1α expression and MVD in lymph nodes with follicular hyperplasia

4. Test possible differences in VEGF-A and HIF1α expression and MVD with patients in described groups

5. Examine possible differences of International Prognostic Index (IPI) in patients with DLBCL and Follicular Lymphoma International Prognostic Index (FLIPI) in patients with low grade Follicular lymphoma with VEGF-A and HIF-1α expression and MVD.

4. MATERIAL AND METHODS

4.1 Patients

The study was prospective and retrospective. Eighty cases (30 DLBCL, 30 FL and 20 FH), diagnosed at the Institutes of Pathology, Faculty of Medicine, University of Pristina, Republic of Kosovo and Faculty of Medicine, Cyril & Methodius University, Skopje, Republic of Macedonia, have been used. No prior therapy had been administered to the study subjects. Reactive lymph node were used as control.

4.2 Immunohistochemical analysis

The histological and immunophenotypical classification was carried out according to the WHO 2008 classification.

Histological specimens, obtained by biopsy, were lymph nodes fixed in 10% neutral buffered formalin (NBF) and embedded in paraffin. Histological sections, i.e., 4μm thick sections were immunohistochemically stained using standard immunoperoxidase techniques. Slides were deparaffinized and rehydrated through a graded ethanol series. Antigen retrieval was carried out by placing the slides in 10 mM sodium citrate buffer (pH 6.0) and boiled 30 min (VEGF-A) or 45 min (CD31 and HIF-1α) on a hot plate, and subsequently allowed to cool for 20 min. Endogenous peroxidase activity was blocked by incubating the sections for 10 min in 3% hydrogen peroxide. The sections were subsequently washed in PBS three times for 5 min. The sections were then incubated overnight at 4^0C with primary antibodies, washed with PBS and then incubated with biotinylated secondary antibodies for 60 min, and 3-amino-9-ethylcarbazole substrate-chromogen (LSAB2 System-HRP; DakoCytomation) for 15 min in the dark for VEGF-A and HIF-1α, and 3,39-diaminobenzidine tetrahydrochloride (DAB, Dako Denmark) for 10 min for CD31. Subsequently, slides were counterstained with haematoxylin. Positive and negative controls were included in each staining run. The rabbit polyclonal antibody anti-VEGF-A (A-20, 1:150 dilution; Santa Cruz Biotechnology Inc) was used for recognizing the N-terminus of VEGF-A of human origin. HIF-1α staining were performed with a rabbit polyclonal antibody (clone H206, dilution 1:25: Santa Biotethnology Inc.,

Santa Cruz, CA, USA). Anti-CD31, a mouse monoclonal antibody (Clone JC70A, RTU; DAKO, Denmark), was used for microvessel staining.

VEGF-A protein expression was analyzed in the cytoplasm of tumor cells, and the immunohistochemical score (IHS) was calculated by combining the quantity score (percentage of positive stained cells) with the staining intensity score. The quantity score was ranked from 0 to 4:

0= no immunoreactivity.

1= <25% cells stained.

2= 26–50% cells stained.

3 =51–75% cells stained.

4=>76% cells stained.

The staining intensity was score-ranked as follows:

0 (negative),

1 (weak positivity, seen at 400x magnification),

2 (moderate, seen at 100x magnification), and

3 (strong, seen at 40x magnification).

HIS, used to correlate VEGF-A expression with prognostic indices (IPI for DLBCL, and FLIPI for FL) was defined as follows: 0 = no immunoreactivity; 1 (low positivity) 1-50% stained and seen at 400x; 2 (moderate positivity) 1-50% cells stained and seen at 40x or >50% of cells stained and seen at 400x; and 3 (high positivity) 51-100% cells stained and seen at 40x.

The nuclear positivity of HIF-1α was analyzed in the nucleus of tumor cells, and the IHS score was calculated by combining the quantity score (percentage of positive stained cells) and the staining intensity score as for VEGF-A.

Angiogenesis assessment was carried out by microvessel count. Immunostained tumor sections were scanned at 20x magnification in order to identify the areas with highest vascular density – so called “hot-spots”. MVD was measured in five fields with a higher density of CD31-positive cells and cell clusters at 200x magnification. The mean value of microvessels density in five examined hot spots per section was then calculated and the MVD median value was used to classify the cases as “high” and “low” MVD. If the mean value was ≤ 10, the case was considered as “low” MVD, while if it was > 10 it was considered as “high” MVD.

The International Prognostic Index (IPI) score for DLBCL was determined as follows.

One point was awarded for each of these characteristics:

1) Age older than 60,

2) Elevated serum LDH,

3) Poor general health status (ECOG performance status score of 2 or greater),

4) Stage III or IV disease, and

5) More than one involved extranodal disease site.

This results in a total score ranging from zero to five. Risk groups are defined as follows:

Low risk (0-1 points)

Low-intermediate risk (2 points)

High-intermediate risk (3 points)

High risk (4-5 points)

Five factors taken into account for computing FLIPI were:

1) Age older than 60,

2) Disease stage III or IV,

3) Five or more tumors nodes detected, or more than four lymph node groups involved,

4) Serum hemoglobin less than 12 g/dl,

5) Elevated serum LDH.

One point was awarded for each of these characteristics. Risk groups were defined as follows:

Low risk (0-1 points)

Intermediate risk (2 points)

High risk (3-5 points).

4.3 Statistical analysis

Statistical analysis was carried out using IBM SPSS Statistics, Version 22, 32-bit Edition, 14-day trial (statistical Package for the Social Sciences, International Business Machines Corp., 1 New Orchard Road, Armonk, New York, United States).

The χ^2 test was used to test for statistical differences or dependency among all categorical variables measured in the study. All test were performed at significance level of p-value less than 0.05.

An informed consent was obtained from all individuals participating in the study.

This study had been approved by the Ethics Committee of the University Clinical Centre of Kosova, Pristina.

5. RESULTS

5.1. General characteristics

This study included 80 patients, 30 with DBCL, 30 with FL grade 1 and 2, and 20 cases with follicular hyperplasia as a control group.

In 42 patients the diagnosed was made at the Institute of Pathology in Pristina, whereas in 38 it was made at the Institute of Pathology in Skopje. Out of these, 41 were male (16 DLBCL, 16 FL and 9 FH) and 39 females (14 DLBCL, 14 FL and 11 FH). The age of study subjects diagnosed with DLBCL was between 23 and 87 years, FL between 29 and 73, and FH between 7 and 66 (Table 2).

Table 1. Description of characteristics of subjects

		Sex		
		Female **39 (48.75%)**	**Male** **41 (51.25%)**	**Total** **80**
Age	Median	55	59	55
	Minimum	7	15	7
	Maximum	87	80	87
	Standard deviation	17.78	18.4	18

5.2. Expression of VEGF-A in Diffuse Large B-cell lymphoma

Regarding quantity score, following results were obtained:

i. One tumor sample had no immunoreactivity (quantity score 0)
ii. Eighteen were Group I (quantity score 1 and 2),
iii. Eleven were Group II (quantity score 3 and 4).

Regarding intensity score, following results were obtained:

i. One tumor sample had no immunoreactivity,
ii. Five showed weak positivity (seen in 200x magnification),
iii. Twelve showed medium intensity (seen in 100x magnification) and
iv. Twelve showed high intensity (seen in 40x magnification)

Table 3 below illustrates this.

Table 2. Expression of VEGF-A in Diffuse Large B-cell lymphoma

	VEGFexpression	
Score	Quantity	Intensity
0	1 (3%)	1 (3%)
1	18 (60%)	5 (17%)
2	11 (37%)	12 (40%)
3		12 (40%)

The analysis indicated that in 60% of the cases 1% to 50% of the cells were stained, while in 37% of the cases over 50% of the cells showed immunoreactivity. In 80% of cases, the staining was of medium to high intensity.

5.3. Expression of HIF-1α in Diffuse Large B-cell lymphoma

Results of the immunohistochemical expression of HIF-1α in the nucleus of tumor cells were as follows:

i. Two cases did not show any immunoreactivity (quantity score 0),
ii. Twenty-three were Group I (quantity score 1 or 2) and
iii. Five were in Group II (quantity score 3 or 4).

As far as the intensity is concerned:

i. Two had no immunoreactivity
ii. Seven were weakly positive (seen at 200x magnification),
iii. Twelve were of a medium intensity (seen at 100x magnification) and
iv. Nine were of high intensity (seen at 40x magnification).

Table 4 below illustrates this distribution.

Table 3. Expression of HIF-1α in diffuse large B-cell lymphoma

	HIF-1α expression	
Score	Quantity	Intensity
0	2 (7%)	2 (7%)
1	23 (77%)	7 (23%)
2	5 (17%)	12 (40%)
3		9 (30%)

In most tumor samples HIF-1α expression was present in 1%-50% of lymphoma cell nuclei, whereas in only 17%, more than 50% of cells stained positive. The intensity of staining did not differ between groups, with a slight majority of those with medium intensity (seen at 100x magnification).

5.4. MVD in diffuse large B-cell lymphoma

Immunohistochemical expression of CD31 was analyzed in order to detect blood vessels or individual endothelial cells. MVD was measured as described in the “Methods” section.

In DLBCL, out of 30 examined cases, high MVD was found in 18 cases, whereas 12 had low MVD.

Table 4. MVD in diffuse large B-cell lymphoma

	MVD in DLBCL
Low	12 (40%)
High	18 (60%)
Total	30 (100%)

At 200x examination, using CD31 expression in five examined hot-spots, 60% of cases with DLBCL have high MVD, or over ten blood vessels positive for expression of CD31 per five hot spots.

5.5. Expression of VEGF-A in low grade follicular lymphoma

No immunoreactivity was noted in five of them (quantity score 0), 22 were in Group I (quantity score 1 and 2) and three in Group II (quantity score 3 and 4).

As far as intensity was concerned, five were without immunoreactivity, five were weakly positive (seen at 200x magnification), fifteen were of medium intensity (seen at 100x magnification) and five showed strong intensity (seen at 40x magnification).

Table 5. Expression of VEGF-A in low grade follicular lymphoma

	VEGF expression in FL	
Score	Quantity	Intensity
0	5 (17%)	5 (17%)
1	22 (73%)	5 (17%)
2	3 (10%)	15(50%)
3		5 (17%)

The results indicate that in 73% of cases between 1% and 50% of tumor cells stained positive for VEGF-A, whereas in only 10% of cases more than 50% cells stained positive.

In half of the cases the stain was of medium intensity, whereas the other half was split in three equal parts between no staining, weak and high intensity staining.

5.6. Expression of HIF-1α in low grade follicular lymphoma

Regarding the quantity score, results were as follows:

i. Four cases showed no of immunoreactivity (quantity score 0),
ii. Twenty-four were in Group I (quantity score 1 or 2) and
iii. Two were in Group II (quantity score 3 or 4).

Regarding the intensity score, results were as follows:

i. Four had no immunoreactivity,
ii. Seven showed weak positivity (seen at 200x magnification),
iii. Fourteen had medium intensity (seen at 100x magnification)
iv. Five had high intensity (seen at 40x magnification)

Table 7 below illustrates this distribution.

Table 6. Expression of HIF-1α in low grade follicular lymphoma

	HIF-1α expression in FL	
Score	Quantity	Intensity
0	4 (13%)	4 (13%)
1	24 (80%)	7 (23%)
2	2 (7%)	14 (47%)
3		5 (17%)

In 80% of FL cases 1%-50% of tumor cell nuclei expressed HIF-1. In only 7% of the cases did more than 50% tumor cells stain positive. As far as the intensity was concerned, the majority of cases had medium staining intensity (seen at 100x magnification).

5.7. MVD in low grade follicular lymphoma

Out of 30 examined cases, 13 had high MVD and 17 low.

Table 7. MVD in low grade follicular lymphoma

	MVD expression in FL
Low	17 (57%)
High	13 (43%)

Using CD31 expression in five examined hot-spot fields, 43% of low-grade FL had high MVD with more than 10 blood vessels per field visible at 200x magnification.

5.8. Expression of VEGF-A in follicular hyperplasia

Regarding the quantity score, results were as follows (Table 9):

i. Five cases were negative (quantity score 0),

ii. Fifteen cases showed some degree of, in all of the cases in less than 50% of cells (quantity score 1 or 2).

The staining intensity correlated with quantity; five cases had no immunoreactivity, six were weakly positive (seen at 200x magnification), seven had medium intensity staining (seen at 100x magnification) and two high intensity (seen at 40x magnification).

Table 8. Expression of VEGF-A in follicular hyperplasia

	VEGF expression in FH	
Score	Quantity	Intensity
0	5 (25%)	5 (25%)
1	15 (75%)	6 (30%)
2	0 (0%)	7(35%)
3		2 (10%)

VEGF-A expression was seen in 75% of FH cases. However, in all of these less than half of lymphoid cells were positive. On the other hand, the staining intensity was spread uniformly across most categories, except for a lower proportion (10%) of cases with high intensity expression.

5.9. Expression of HIF-1α in follicular hyperplasia

Regarding the quantity score, results were as followed:

i. Six showed no immunoreactivity (quantity score 0),
ii. All fourteen cases showing immunoreactivity were of Group I (quantity score 1 or 2),
iii. None of the cases was in Group II (quantity score 3 or 4).

Results of staining intensity score were as follows:

i. Six showed no immunoreactivity,
ii. Eleven were weakly positive (seen at 200x magnification)
iii. Three had medium intensity staining (seen at 100x magnification)
iv. None had high intensity staining (seen at 40x magnification).

Table 9. Expression of HIF-1α in follicular hyperplasia

	HIF-1α expression in FL	
Score	Quantity	Intensity
0	6 (30%)	6 (30%)
1	14 (70%)	11 (55%)
2	0 (0%)	3 (15%)
3		0 (0%)

In contrast to the other two groups, 30% of the FH cases were HIF-1α negative, whereas in the proportion of positive cells was less than 50%. All positive cases stained weakly or with moderate intensity.

5.10. MVD in follicular hyperplasia

Sixteen of twenty cases had high MVD (more than 10 blood vessels visible per field at 200x magnification) and only four low MVD (table 11).

Table 10. MVD in follicular hyperplasia

	MVD in FH
High	16 (80%)
Low	4 (20%)

5.11. Differences in VEGF-A expression between DLBCL, FL, and FH

We compared VEGF-A expression between DLBCL, an aggressive lymphoma, follicular lymphoma, an indolent lymphoma, and follicular hyperplasia, a benign condition. The difference in the proportion of positive cells was statistically significant (p=0.001), with DLBCL having most and FH least positive cells. In contrast, there was no difference in the intensity of positivity among groups (p=0.118).

Table 11. Differences in VEGF-A expression between DLBCL, FL, and FH

VEGF-A Score	DLBCL		FL		FH	
	Quantity	Intensity	Quantity	Intensity	Quantity	Intensity
0	1	1	5	5	5	5
1	17	5	23	4	15	6
2	12	12	2	11	0	7
3		12		10		2
Quantity: χ^2= 19.609; DF = 4, p-value = 0.001						
Intensity: χ^2=10.149; DF = 6, p-value = 0.118						

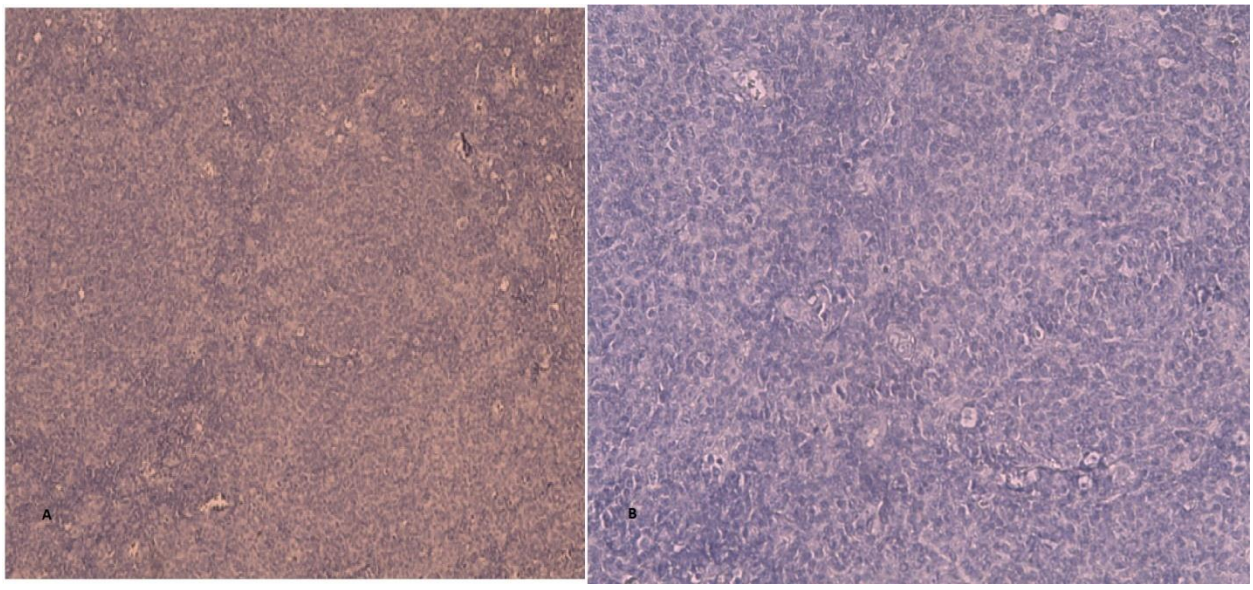

Figure 6. Immunohistochemical staining of FL for VEGF-A. Reaction is negative. A. 100x, B. 200x.

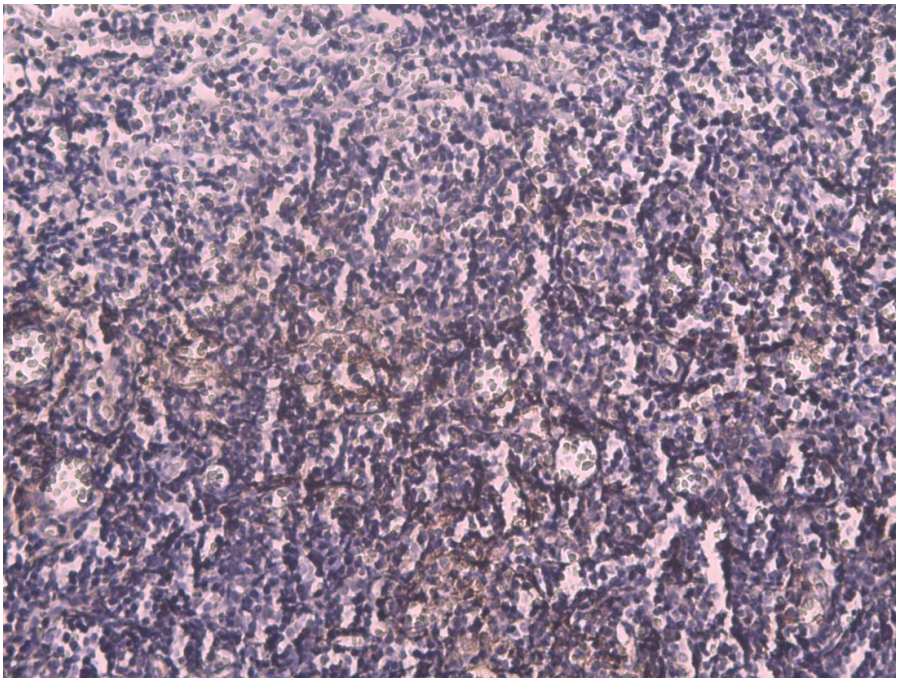

Figure 7. Immunhistochemical staining of FL for VEGF-A. Reaction is positive, quantity score 1 (Magnification 200x).

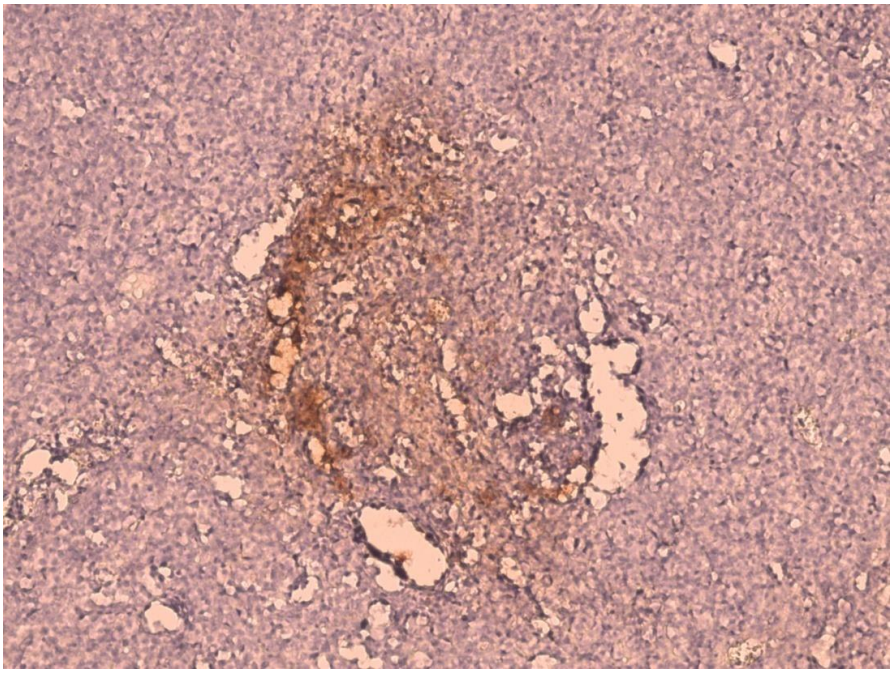

Figure 8. Immunohistochemical staining of FL for VEGF-A. Quantity score 2 (100x)

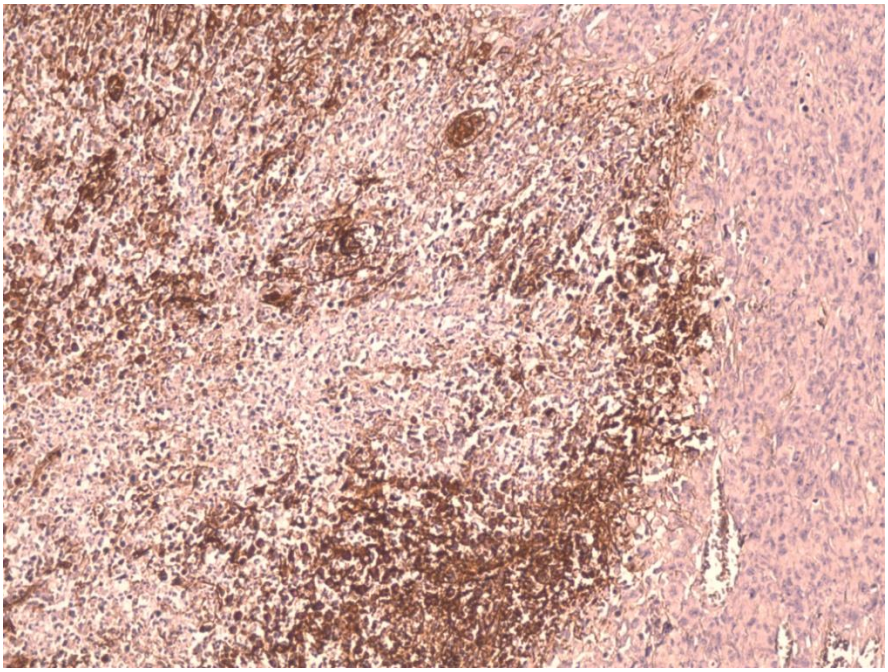

Figure 9. Immunohistochemical staining of DLBCL for VEGF-A. Quantity score 3, Intensity 3 (100x)

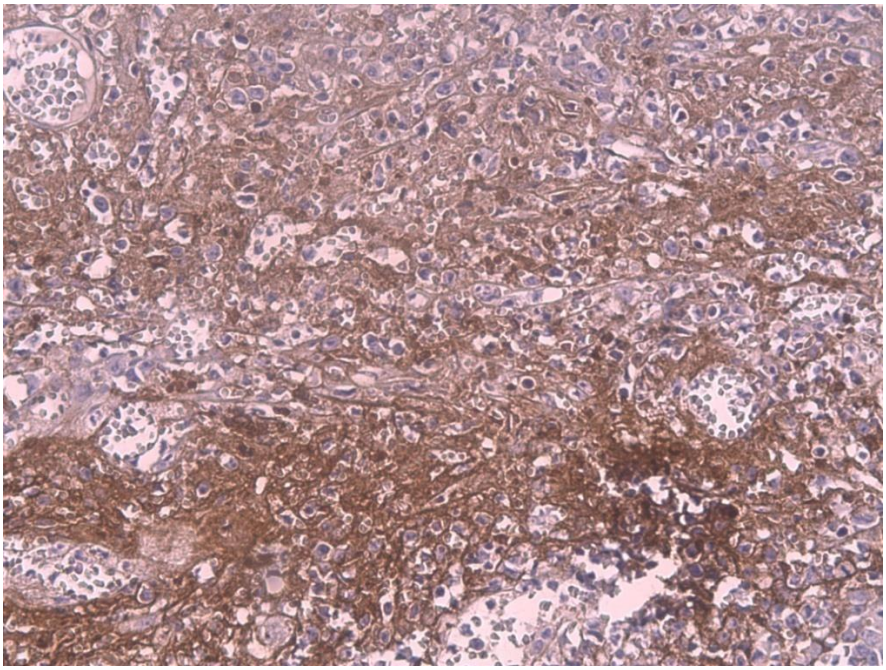

Figure 10. Immunohistochemical staining of DLBCL for VEGF-A. Quantity score 4, Intensity 3

5.12. Differences in HIF-1α expression between DLBCL, FL and FH

Regarding the expression of HIF-1α, the difference in the proportion of positive cells was statistically significant (p=0.01), with DLBCL having most and FH least positive cells. In contrast, there was no difference in the intensity of positivity among groups (p=0.18).

Table 12. Differences in HIF-1α between DLBCL, FL and FH

HIF-1α score	DLBCL		FL		FH	
	Quantity	Intensity	Quantity	Intensity	Quantity	Intensity
0	2	2	4	4	6	6
1	23	7	26	5	14	6
2	5	12	0	15	0	6
3		9		6		2
Quantity: χ^2= 13.228, DF = 4, p-value = 0.010						
Intensity: χ^2= 8.883, DF = 6, p-value = 0.180						

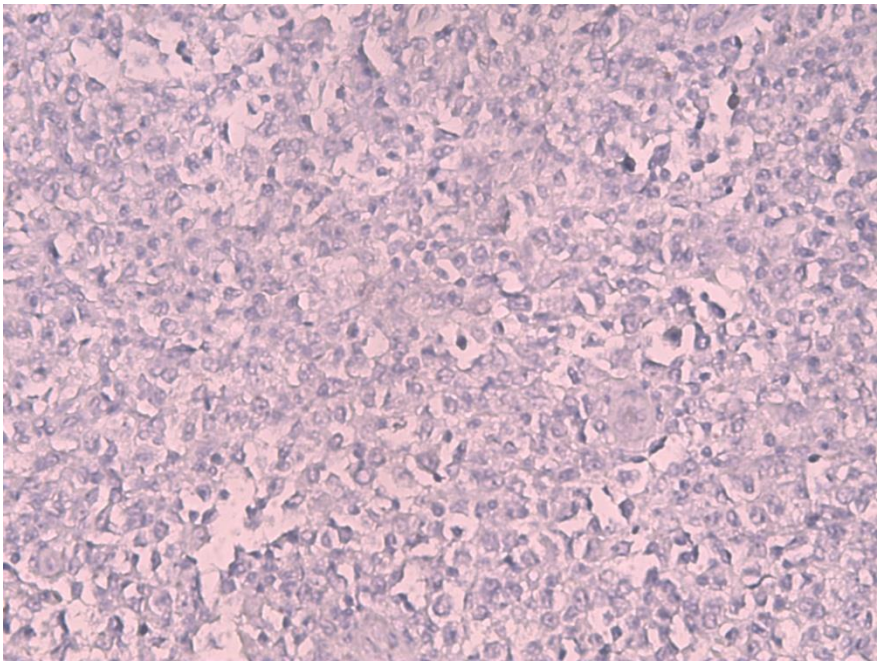

Figure 11. Immunohistochemical staining of FL for HIF-1α. Quantity score 0. (200x)

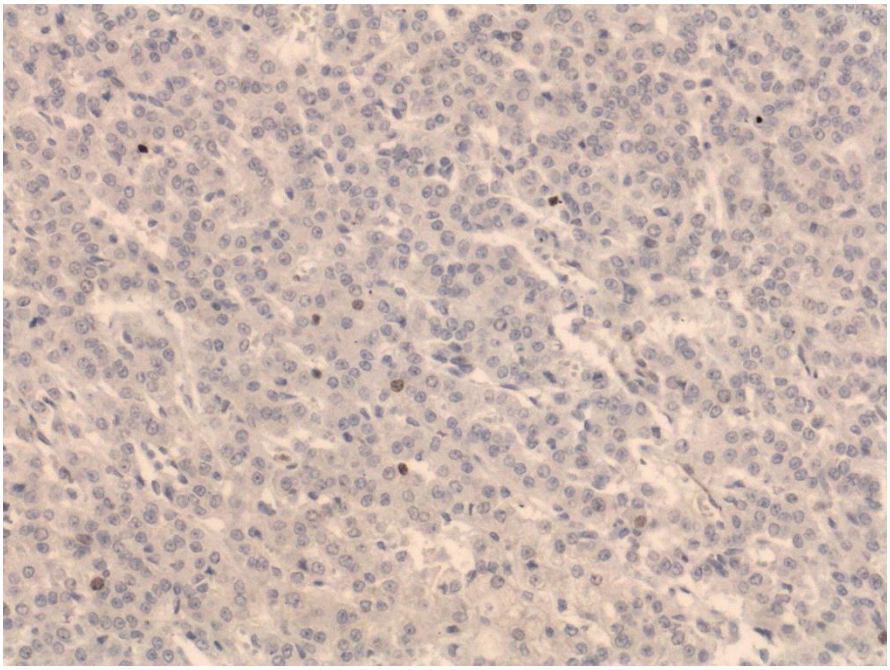

Figure 12. Immunohistochemical staining of FL for HIF-1α. Quantity score 1, intensity score 2. (100x)

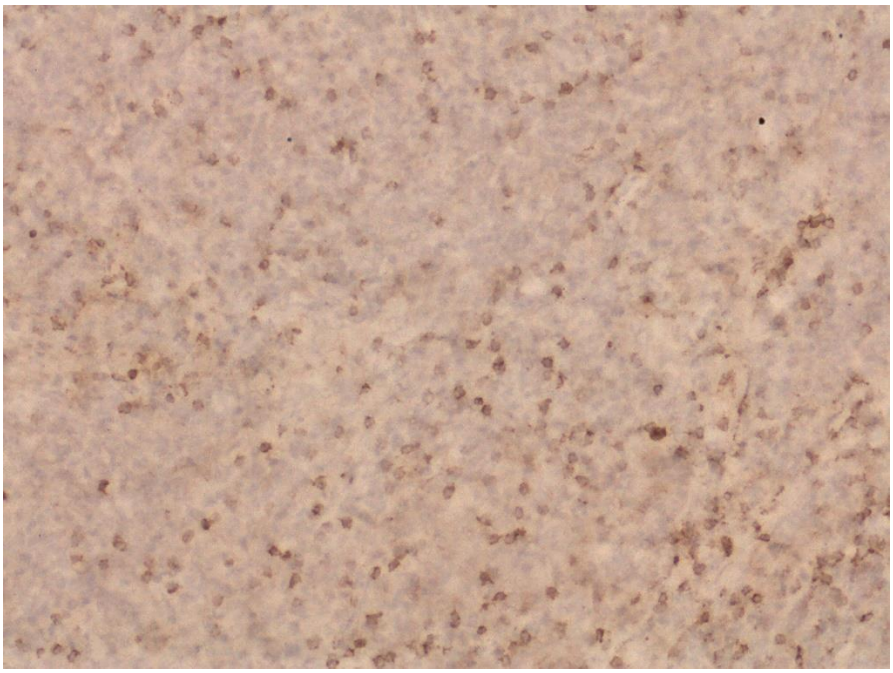

Figure 13. Immunohistochemical staining of DLBCL for HIF-1α. Quantity score 2, intensity score 2. (100x)

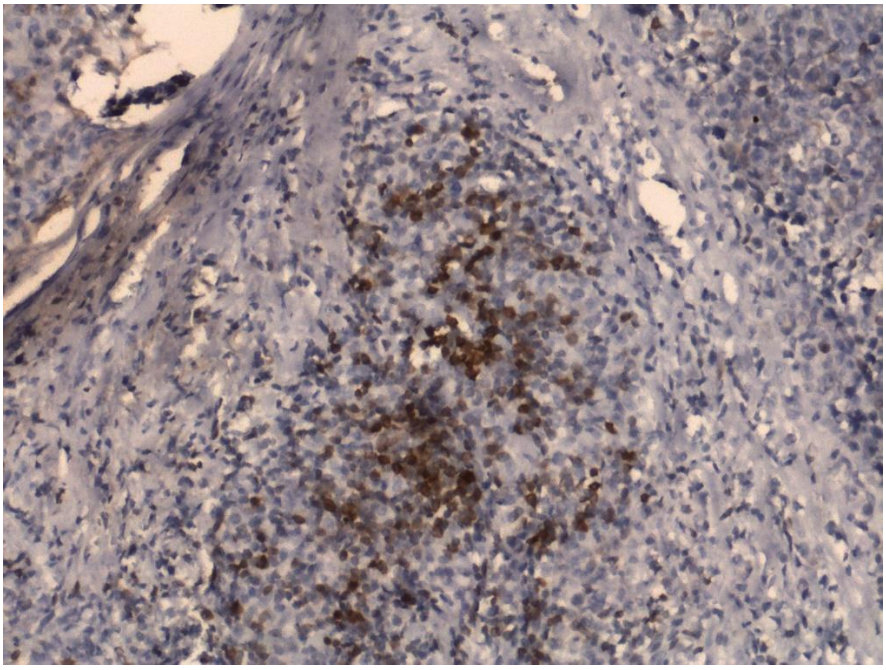

Figure 14. Immunohistochemical staining of DLBCL for HIF-1α. Quantity score 3, intensity score 3. (100x)

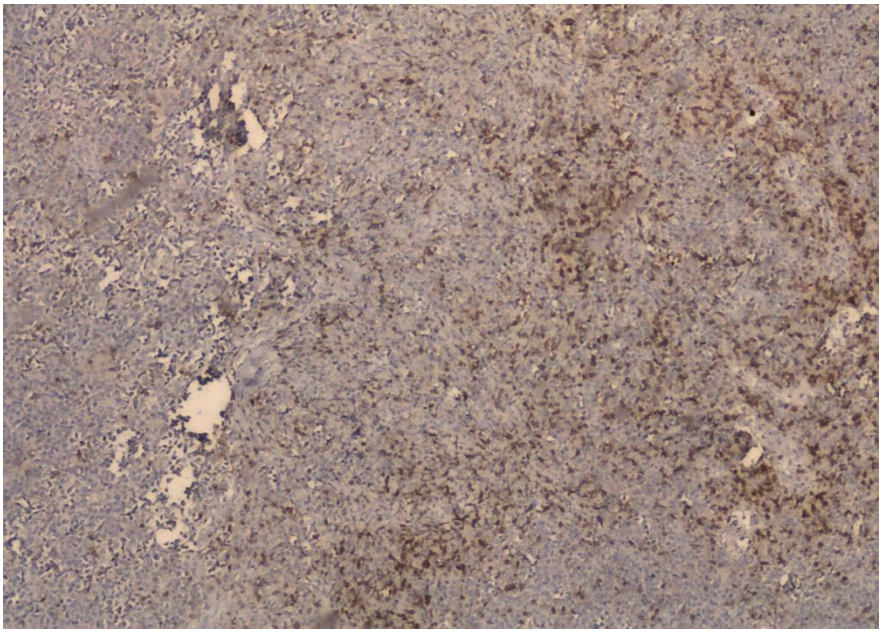

Figure 15. Immunohistochemical staining for HIF-1α. Quantity score 4, intensity score 3. (40x)

5.13. Differences in MVD between DLBCL, FL and FH

Regarding MVD, the difference between groups was statistically significant (p=0.035) (table 14). However, highest MVD was seen in FH, and lowest in FL.

Table 13. Differences in MVD between DLBCL, FL and FH

MVD score	DLBCL	FL	FH
High	18	13	16
Low	12	17	4
Quantity: χ^2= 6.688, DF = 2, p-value = 0.035			

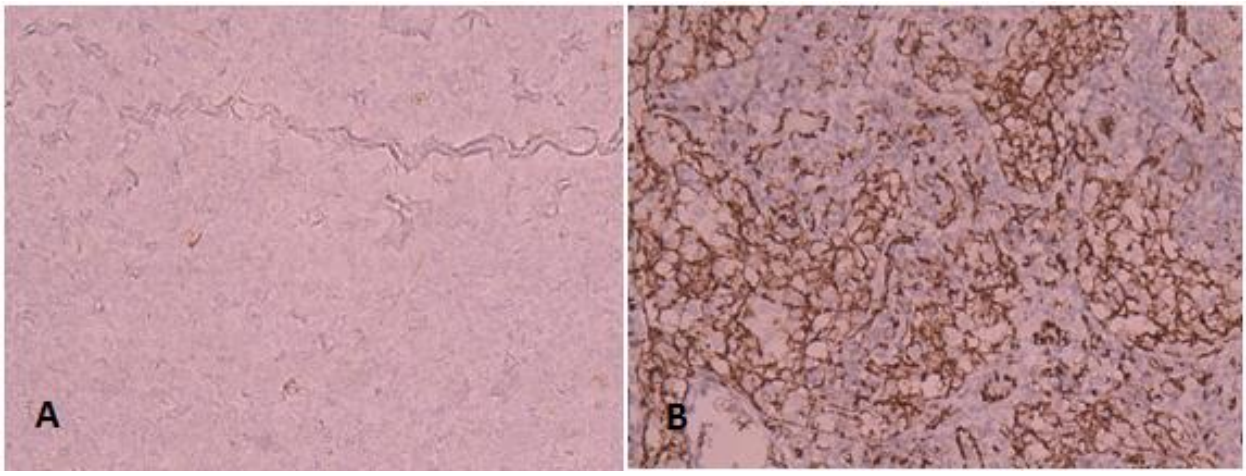

Figure 16. CD31 staining of DLBCL (A) Low microvessel density and (B) High microvessel density at 40X.

5.14. IPI and VEGF-A IHC in DLBCL

In patients with DLBCL, higher VEGF-A IHC, calculated as described in the “Methods” section, was more frequently present in patients with higher IPI (p=0.013) (table 15).

Table 14. International Prognostic Index in DLBCL and Vascular Endothelial Growth Factor-A IHS

	IPI		
VEGF-A	Low	Intermediate	High
0	1	0	0
1	3	2	0
2	4	11	0
3	0	3	6
χ^2= 6.136, DF = 1, p-value = 0.013			

5.15. FLIPI and VEGF-A IHC in FL

Similarly, higher VEGF-A IHC was more frequent in patients with FL with higher FLIPI values (p=0.035) (table 16).

Table 15. Differences between FLIPI in FL and Vascular Endothelial Growth Factor-A

	FLIPI		
VEGF-A	Low	Intermediate	High
0	4	1	0
1	3	1	0
2	5	9	4
3	0	1	2
χ^2= 6.688, DF = 2, p-value = 0.035			

5.16. IPI and HIF-1α IHC in DLBCL

Despite the fact that both negative cases for HIF-1α expression were in the low or intermediate IPI category and no patient with low IPI had high HIF-1α IHC, the difference in HIF-1α IHC values between DLBCL patients with different IPI scores was not statistically significant (p=0.149) (table 17).

Table 16. Differences of IPI in DLBCL with Hypoxia Inducible Factor-1α

	IPI		
HIF-1α	Low	Intermediate	High
0	1	1	0
1	3	3	1
2	4	9	1
3	0	3	4
χ^2 = 2.078, DF = 1, p-value = 0.149			

5.17. FLIPI and HIF-1α IHC in FL

Patients with FL with higher FLIPI scores had significantly higher HIF-1α IHC (p=0018) (table 18). None of the patients with low FLIPI had HIF-1α IHC 3, and none of the patients with high FLIPI had HIF-1α lHC 0 or 1.

Table 17. Differences of FLIPI in FL with Hypoxia Inducible Factor-1α

	FLIPI		
HIF-1α	Low	Intermediate	High
0	3	1	0
1	3	1	0
2	6	9	4
3	0	1	2
χ^2 = 5.568, DF = 1, p-value = 0.018			

5.18. IPI and MVD in DLBCL

The difference in MVD in patients with different IPI scores was highly statistically significant ($p<0.001$) (table 19). None of eight patients with a low IPI score had high MVD, in contrast to five out of six with high IPI.

Table 18. Differences of IPI in DLBCL with Microvessel Density

	IPI		
MVD	Low	Intermediate	High
1	8	3	1
2	0	13	5
$\chi^2 = 16.372$, DF = 2, p-value < 0.001			

5.19. FLIPI and MVD in FL

In contrast to DLBCL, there was no difference in MVD between FL patients with different FLIPI scores (p=0.311) (table 20).

Table 19. Differences of FLIPI in FL with Microvessel Density

	FLIPI		
MVD	Low	Intermediate	High
1	8	5	4
2	4	7	2
χ^2 = 2.234, DF = 2, p-value = 0.311			

6. DISCUSSION

Results of this study demonstrated a high percentage of VEGF-A expression in DLBCL. In 97% of cases there was positive expression of VEGF-A. There is abundant evidence from clinical, morphological and molecular-genetic studies that DLBCL is heterogenous disease (116, 117). It was demonstrated in a lot of studies that expression of VEGF-A is in connection with aggressiveness of disease in solid tumors (39, 118, 119). Recently, a similar relationship has been described in several hematologic malignancies. Expression of the angiogenic peptides vascular endothelial growth factor (VEGF) and basic fibroblast growth factor correlates with clinical characteristics in leukemia and non-Hodgkin's-lymphoma and the serum/plasma concentrations serve as predictors of poor prognosis (120).

When compared with FL and follicular hyperplasia, VEGF-A expression was significantly higher in DLBCL. Such findings verify hypothesis that angiogenesis has close connection with aggressiveness of disease.

Important advancements in angiogenesis as a target for treatment against cancer were reported in studies of Folkman et al., who have used endostatin and angiostatin for their experimental studies in treatment in rats (121). From different studies, now is known that hypoxia drives angiogenesis and, in this way, affects development, extent and prognosis of tumors (122). Tumor microenvironment has been recognized to influence neoplastic progression and growth. Angiogenesis is an important mediator of tumor progression. As tumors expand, diffusion distances from the existing vascular supply increases resulting in hypoxia. Sustained expansion of a tumor mass requires new blood vessel formation to provide rapidly proliferating tumor cells with an adequate supply of oxygen and metabolites (114).

Some authors also reported a positive correlation between neovascularization and tumor grade according to several lymphoma classification systems (23). Zhao et al., found that VEGF-A levels were significantly higher in lymphoma than in follicular hyperplasia (123). Our results showed similar trend in VEGF-A expression in DLBCL, FL and FH.

Environment inside even a small tumor is characterized by total (anoxia) or partial oxygen deprivation, (hypoxia). Solid tumors contain regions with mild (hypoxia) to severe oxygen deficiency (anoxia), due to lack of blood supply to the growing tumor nodules (125, 126). A recent report by Gratzinger et al., showed that high VEGF and VEGFR-1 protein expression in DLBCL was associated with significantly improved PFS and OS. They noted that VEGF and its receptors are expressed at high levels in a subset of DLBCL, and that the combination of high VEGF and high VEGFR-1 expression by lymphoma cells (denoted VEGF+R1) is predictive of both overall and progression-free survival independent of the IPI. Incorporating VEGFR-2 status does not improve prognostic value with regard to survival. This raises the question of autocrine signaling via a self-contained VEGF-VEGFR-1 pathway in a subset of DLBCLs (55).

Expression of HIF-1α in our study has shown high expression in cases with DLBCL. Most cases expressed HIF-1α and its expression was significantly higher in DLBCL than in FL and FH. This is in accordance with previous studies that aggressive tumors are hypoxic. Lowest percentage of HIF-1α was founds in control cases of follicular hyperplasia.

The prime driving force for tumor angiogenesis is hypoxia, and HIFs play a key role in this process. Growing evidence supports that HIFs are involved in angiogenesis. HIF expression was directly correlated with microvessel density and cyclooxygenase 2 expression in many cancers, indicating its potential role in angiogenesis of carcinomas (86, 109, 124).

Evens et al. found that patients with DLBCL treated with R-CHOP had superior outcome if HIF-1α was expressed. This HIF-based survival difference was not apparent in CHOP-treated patients. The reason for this is not clear, but there are several possible explanations (110).

Expression of CD31used for evaluation of MVD in our study was higher in DLBCL than in FL. Such findings are in accordance with studies that determine MVD as an important prognostic factor in aggressive malignant tumor.

Our results were also comparable to those that exhibit a high expression of CD31 in follicular hyperplasia, compared with aggressive or indolent lymphomas. Data obtained from this study was statistically significant and even more expressed than in previously reported results.

In most tumor types, increased MVD correlates with increased disease progression and decreased survival. Similar studies have been reported in hematopoietic tumors, including multiple myoeloma and lymphoma (36, 69, 71, 127, 128).

Wobser et al., showed that a proangiogenic micromilieu is associated with a worse prognosis in systemic lymphoma. Hence, targeting the tumor microenvironment and its vasculature has evolved as a promising novel treatment strategy (129). Authors found that primary cutaneus B-cell lymphoma-leg type (PCLBCL-LT) with higher mean baseline MVD (CD31+) exhibited a slightly worse prognosis; however, without statistically significant difference.

The contribution of histopathological microvessel density to survival in diffuse large B-cell lymphomas treated with immunochemotherapy remains unknown. Cardesa-Salzmann et al., showed 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 showed a significantly poorer PFS and OS than those with a low MVD (117).

The contribution of microvessel density to follicular lymphoma survival remains controversial (127). Koster *et al.,* showed increased MVD to be associated with a more favorable OS in a series of 36 uniformly treated patients given CVP and interferon (IFN) α2b followed by IFN maintenance(69). Jorgensen *et al.,* analyzed 107 FL cases with heterogeneous treatments and found increased interfollicular MVD predicted inferior OS and increased transformation to DLBCL(27). Controversial results reported Farinha et al., in their study. They showed that increased angiogenesis is an independent marker of inferior survival and may promote transformation (127).

However, MVD in reactive nodes has been found to be higher (Ridell et al) or comparable (Arias et al) to that observed in lymphomas, including large cell lymphomas (59, 60).

From our study, we found that there is a positive correlation with stage of disease and expression of VEGF-A. High expression of VEGF-A was found in cases with advanced disease, while in cases with low IPI, also VEGF-A was low. Based on these results we can expect in most advanced cases to be used antiangiogenic factors as a treatment.

Although the International Prognostic Index (IPI) or revised IPI have been used as the standard clinical tool to predict outcomes, outcomes differ significantly within IPI categories. Therefore, new biological markers that reflect the heterogeneity of DLBCL have been evaluated to better determine patient outcomes (130, 131, 132). Angiogenesis is a fundamental process in the growth and metastatic dissemination of both solid tumors and hematologic malignancies (133). Dou et al., found that serum levels of angiogenic factor VEGF are related to the development and progression of DLBCL. The VEGF combined with IPI can be used for evaluating the prognosis of DLBCL (134). Ganjo et al., revealed that VEGF-C is correlated with LDH and IPI score, but not with progression-free or overall survival in DLBCL (106).

Our study shows that cases with high IPI have high expression of HIF-1α, while in cases when disease is more limited (low IPI) than HIF-1α expression was also lower. Evens et al., found that HIF-1α expression remained a significant independent factor predicting for improved PFS and OS, whereas the IPI was significant for PFS and of borderline significance for OS (110).

Powell et al., showed by multivariate regression analysis of IPI criteria and either HIF-1α expression, VEGF expression, or HIF-1α and VEGF co-expression according to the Cox proportional hazards model that there was no significant association between HIF-1 α or VEGF expression and overall survival or progression free survival (135). Salzmann et al., in the final model with 111 cases, reported that both IPI and MVD maintained independent prognostic importance for OS. Patients with high MVD had shorter PFS

than those with low MVD values. Patients with high MVD showed significantly shorter OS in comparison to those with low MVD (117).

Our results are in line with most of other reported results until now. We have shown that patients with high IPI have also high MVD and vice versa. Increased MVD has also been described as a negative prognostic factor in solid tumors (136). The presence of tumor associated angiogenesis may alter the outcome of patients with DLBCL and could be a prognostic factor. Further clinical studies are needed to correlate the degree of angiogenesis with response to anti-angiogenesis agents (106).

Gratzinger et al., found that increasing microvessel density is a poor prognostic indicator for overall but not progression-free survival, and is independent of the IPI. Microvessel density is statistically significant only when computed as a continuous variable over the whole range of vascularity. A specific cut-point that would risk–stratify patient populations with statistical significance is lacking. Although increased vascularity may be associated with clinical aggressiveness in DLBCL, this association could also represent an epiphenomenon reflecting high circulating levels of angiogenic growth factors and inflammatory cytokines which identify a patient population at risk for poor outcome (55).

Compared with other research, our results of association between FLIPI group in FL and expression level of VEGF-A show that VEGF-A and FLIPI could be important prognostic factors in follicular lymphoma. Except different prognostic factors studied from other authors in prognosis and treatment of FL, also FLIPI and VEGF-A can be considered as an important factor in determination of prognosis and outcome of disease.

De Mendonca et al., reported that FLIPI score and *VEGFA*-2578 C>A predicted PFS and OS, and bulky disease influenced only OS (137). Lozano-Santos et al., analyzed the contribution of *VEGFA* genetics in the clinical outcome of the leukemia. Results suggest a potential clinical utility of *VEGFA* genetics as predictors of reduced survival in patients with indolent leukemia and would allow to further refine the classification of this group of CLL patients. The implication of the presence of increased vascularity in the

bone marrow has been described in the outcome of various hematological diseases. This led to the study of the clinical significance of *VEGFA* genetics in other hematologic malignancies (138). Aguayo et al., analyzed vascularity and angiogenesis in leukemia and MDS, their data suggest that vascularity and angiogenic factors are increased in leukemia and MDS and may play a role in the leukemogenic process (139).

Despite evidence for a vital role of angiogenesis in supporting tumorigenesis, some reports have suggested that increased vessel density correlates with better prognosis in FL. Intriguing results by Streubel et al demonstrated that endothelial cells of the microvasculature share the BCL2 rearrangement characteristic of FL, implying an intimate relationship between the two (140).

Except other prognostic factors described from different authors, in the case of our comparison between expression of HIF-1α and FLIPI in follicular lymphoma it is seen that both these factors are in correlation and from this we can consider that with advancement of the disease also increases hypoxia in tissue.

Several solid tumors demonstrate constitutive HIF activation and elevated HIF levels in solid tumors have been linked to poor prognosis (104, 141). Evens et al., reported in their study that it is possible that this observation is related to the more aggressive nature of DLBCL compared with FL (105). Evens et al., in one study examined the relationship of HIF-1α protein expression with clinical outcome. When HIF-1α protein was analyzed as a dependent variable (positive *vs* negative) among all patients, there was a trend toward improved survival for HIF-1α–positive patients. When analyzed according to treatment group, there was no difference noted in PFS or OS among patients who received CHOP (110).

The development of hypoxic conditions during the progression of solid tumors was very well documented (25, 100, 142), and was shown to be mediated through a complex network of signaling including HIF-dependent and -independent pathways (83, 143, 144). Targeting hypoxia is an emerging strategy for the treatment of hematologic malignancies, in which hypoxia-induced cellular adaptation mechanisms and hypoxic cells are being used as therapeutic targets for prevention of angiogenesis, metastasis,

stemness, and drug resistance (83). Muz et al., reported that tumor-hypoxia was shown to develop in correlation with the progression of hematologic malignancies and is associated with aggressive tumor behavior. Exploiting the distinctive features of hypoxia and hypoxia-regulated molecules in hematologic malignancies is a promising strategy to improve the treatment of hematologic malignancies; however, its full potential is yet to be determined (83).

The intensity of HIF-1α expression seen in the lymphoma cases is weaker than that seen in other tumor types including colon, lung, renal and breast carcinomas (104, 145, 146).

Stewart et al., reported that follicular lymphomas showed surprisingly little HIF-1α staining, when compared with reactive tissues. This is in contrast to the majority of human cancers in which HIF-1α is over-expressed as a result of intratumoral hypoxia and oncogenic mutations (144, 147).

In our study, MVD levels and FLIPI stages in FL showed no statistical differences. As in the case with low MVD also in those with high MVD, there was equal distribution of FLIPI between groups. Taskinen et al., in their study reported that overall survival and progression-free survival are significantly better among patients with low CD31+ MVDs. CD31+ MVD had prognostic value independently of FLIPI (148). Koster et al., reported similar results. They found that high MVD, as a variable of increased tumor vascularization, was associated with a significantly more favorable outcome in terms of both progression-free and overall survival (70).

7. CONCLUSION

The general hypothesis of the doctoral dissertation was: DLBCL is characterized by higher expression of VEGF-A, HIF-α and higher MVD than low grade follicular lymphoma. This hypothesis was proven by our results.

The general aim of this doctoral dissertation was to evaluate the link between hypoxia and the regulation of angiogenesis in diffuse large B-cell lymphoma and low grade follicular lymphoma. From our results, the following conclusions can be drawn:

1. Expression of VEGF-A is higher in DLBCL, followed by FL, while it is lowest in FH.

2. Expression of HIF1α is also higher in DLBCL, than in FL, and much lower in FH.

3. MVD is higher in FH than in DLBCL, while FL had the lowest percentage of cases with high MVD.

4. DLBCL patients with higher IPI have higher expression of VEGF-A, higher MVD, but not HIF1α.

5. FL patients with higher FLIPI have higher expression of VEGF-A, and HIF-1α, but there are not found correlation between FLIPI in FL and MVD.

8. ABSTRACT

Angiogenesis is essential for the development, growth and progression of tumors. It is induced by hypoxia through mechanism that include the angiogenic transcription factor, hypoxia-inducible factor (HIF). Vascular endothelial growth factor (VEGF) is a well-known proangiogenic factor whose impact on tumor biology is widely studied.

The aim of this study was to evaluate VEGF-A, HIF-1α expression and microvessel density (MVD) in diffuse large B-cell lymphoma (DLBCL), the most frequent aggressive lymphoma type, low grade follicular lymphoma (FL), the most frequent indolent lymphoma type, and reactive follicular hyperplasia (FH), a benign condition with lymphadenopathy.

These factors were analyzed in 80 patients (30 DLBCL, 30 FL and 20 FH) diagnosed at the Institutes of Pathology, Faculty of Medicine, University of Pristina and Faculty of Medicine, Cyril & Methodius University of Skopje. VEGF-A and HIF-1α expression was determined using immunohistochemistry, percentage of positive tumor cells and staining intensity were analyzed. MVD was determined by counting the number of blood vessels identified by antiCD31 immunohistochemistry in hot-spots as well as the total number of CD31 positive endothelial cells. The results were compared with Follicular Lymphoma International Prognostic Index – FLIPI (for FL) and International Prognostic Index – IPI (for DLBCL) scores.

Our results confirm that angiogenesis and hypoxia correlate with aggressiveness of lymphoma. DLBCL had higher expression of VEGF-A, HIF-α and higher MVD than FL. Also, VEGF-A expression and MVD correlated with IPI and VEGF-A and HIF-1α expression with FLIPI.

9. SAŽETAK

Angiogeneza je ključna za razvoj, rast i progresiju solidnih tumora. Potaknuta je hipoksijom kroz mehanizam koji uključuje angiogenetski transkripcijski faktor hypoxia-inducible factor (HIF). Vaskularni enodotelijalni faktor rasta (VEGF) dobro je poznat proangiogeni faktor, čija se uloga u biologiji tumora široko istražuje.

Cilj ovog istraživanja je procijeniti ekspresiju VEGF-A, HIF-α i CD31 (MVD) u difuznom B-velikostaničnom non-Hodgkin limfomu (DLBCL) kao najčešćem agresivnom tipu limfoma, folikularnom limfomu niskog gradusa (FL) kao najčešćem indolentnom tipu limfoma, i u benignom povećanju limfnog čvora - reaktivnoj folikularnoj hiperplaziji (FH).

Nabrojeni faktori analizirani su u 80 slučajeva (30 DLBCL, 30 FL i 20 FH) dijagnosticiranih u Zavodu za patologiju Medicinskog fakulteta Sveučilišta u Prištini i Medicinskom fakultetu Sveučilišta Čirila i Metoda u Skopju. Ekspresija VEGF-A i HIF-α je analizirana nakon imunohistokemijskog bojanja preparata, procjenom postotka pozitivnih tumorskih stanica i intenziteta bojenja. Protutijelom CD31 je procjenjivana gustoća (broj) krvnih žila unutar tumora, kao i ukupni broj CD31 pozitivnih endotelnih stanica. Dobiveni rezultati su uspoređivani s Internacionalnim prognostičkim indeksom za folikularni limfom – FLIPI i Internationalnim prognostičkim indeksom - IPI za DLBCL.

Provedeno istraživanje dokazalo je povezanost hipoksije i angiogeneze s agresivnošću limfoma. DLBCL karakterizira viša razina ekspresije VEGF-A, HIF-α i viši MDV u odnosu na folikularni limfom niskog gradusa. Visoka razina ekspresije VEGF-A, HIF-α i viši MVD također su u korelaciji s visokim IPI (u DLBCL-u) dok su VEGF-A i HIF-α u korelaciji s FLIPI (u FL).

10. REFERENCES

1. Stein H, Warnke RA, Chan WC, Jaffe ES, Chan JKC, Gatter KC, et al. Diffuse large B-cell lymphoma, not otherwise specified. In: Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, et al., ed. WHO Classification of Tumors of Haematopoietic and Lymphoid Tissues. 4th ed. Lyon; 2008.
2. Abramson JS, Shipp MA. Advances in the biology and therapy of diffuse large B-cell lymphoma: moving toward a molecularly targeted approach. Blood. 2005 Aug 15;106(4):1164-74.
3. Martelli M, Ferreri AJ, Agostinelli C, Di Rocco A, Pfreundschuh M, Pileri SA. Diffuse large B-cell lymphoma. Crit Rev Oncol Hematol. 2013,87(2):146-71.
4. Pileri SA, Dirnhofer S, Went P, Ascani S, Sabattini E, Marafioti T, et al. Diffuse large B-cell lymphoma: one or more entities? Present controversies and possible tools for its subclassification. Histopathology. 2002 Dec;41(6):482-509.
5. Puvvada S, Kendrick S, Rimsza L. Molecular classification, pathway addiction, and therapeutic targeting in diffuse large B cell lymphoma. Cancer Genet. 2013 Aug;206(7-8):257-65.
6. Friedberg JW, Fisher RI. Diffuse large B-cell lymphoma. Hematol Oncol Clin North Am. 2008 Oct;22(5):941-52.
7. Ziepert M, Hasenclever D, Kuhnt E, Glass B, Schmitz N, Pfreundschuh M, et al. Standard International prognostic index remains a valid predictor of outcome for patients with aggressive CD20+ B-cell lymphoma in the rituximab era. J Clin Oncol. 2010 May 10;28(14):2373-80.
8. Sehn LH, Berry B, Chhanabhai M, Fitzgerald C, Gill K, Hoskins P, et al. The revised International Prognostic Index (R-IPI) is a better predictor of outcome than the standard IPI for patients with diffuse large B-cell lymphoma treated with R-CHOP. Blood. 2007 Mar 1;109(5):1857-61.
9. The International Non-Hodgkin's Lymphoma Prognostic Factors Project. A predictive model for aggressive non-Hodgkin's Lymphoma. N Eng J Med. 1993 Sep 30;329(14):987-94.

10. Zhou Z, Sehn LH, Rademaker AW, Gordon LI, Lacasce AS, Crosby-Thompson A, et al. An enhanced International Prognostic Index (NCCN-IPI) for patients with diffuse large B-cell lymphoma treated in the rituximab era. Blood 2014 Feb 6;123(6):837-42.
11. Harris NL, Swerdlow SH, Jaffes ES, Ott G, Nathwani BN, De Jong D, et al. Follicular lymphoma. In: Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, et al., ed. WHO Classification of Tumors of Haematopoietic and Lymphoid Tissues. 4th ed. Lyon; 2008.
12. Smith SM. Dissecting follicular lymphoma: high versus low risk. Hematol Am Soc Hematol Educ Program. 2013;2013:561-7.
13. Horn H, Schmelter C, Leich E, Salaverria I, Katzenberger T, Ott MM, et al. Follicular lymphoma grade 3B is a distinct neoplasm according to cytogenetic and immunohistochemical profiles. Haematologica. 2011 Sep,96(9):1327-34.
14. Solal-Céligny P, Roy P, Colombat P, White J, Armitage JO, Arranz-Saez R, et al. Follicular lymphoma international prognostic index. Blood. 2004 Sep 1,104(5):1258-65.
15. A clinical evaluation of the International Lymphoma Study Group classification of non-Hodgkin's lymphoma. The Non-Hodgkin's Lymphoma Classification Project. Blood. 1997 Jun 1;89(11):3909-18.
16. Federico M, Bellei M, Marcheselli L, Luminari S, Lopez-Guillermo A, Vitolo U, et al. Follicular lymphoma international prognostic index 2: a new prognostic index for follicular lymphoma developed by the international follicular lymphoma prognostic factor project. J Clin Oncol. 2009 Sep 20;27(27):4555-62.
17. Arcaini L, Merli M, Passamonti F, Rizzi S, Ferretti V, Rattotti S, et al. Validation of follicular lymphoma international prognostic index 2 (FLIPI2) score un an independent series of follicular lymphoma patients. Br J Haematol. 2010 May;149(3):455-7.
18. Maeng CH, Ahn SW, Ryu SY, Han S, Ko YH, Ji JH, et al. Treatment outcomes and clinical relevance of the Follicular Lymphoma International Prognostic Index in Korean Follicular lymphoma patients treated with chemotherapy. Korean J Intern Med. 2016 May;31(3):560-9.

19. Ganti AK, Bociek RG, Bierman PJ, Enke CA, Vose JM, Armitage JO. Follicular lymphoma: expanding therapeutic options. Oncology (Williston Park). 2005 Feb;19(2):213-28.
20. Jain RK. Tumor angiogenesis and accessibility: role of vascular endothelial growth factor. Semin Oncol. 2002 Dec;29(6 Suppl 16):3-9.
21. Aggarwal D, Srivastava G, Gupta R, Pant L, Krishan G, Singh S. Angiogenesis in Non-Hodgkin's Lymphoma: An Intecategory Comparison of Microvessel Density. ISRN Hematol. 2012;2012:943089.
22. Ruan J, Hajjar K, Rafii S, Leonard JP. Angiogenesis and antiangiogenic therapoy in non-Hodgkin's lymphoma. Ann Oncol. 2009 Mar;20(3):413-24.
23. Ozbudak IH, Akkaya B. Karpuzoğlu G. Vascular endothelial growth factor expression in low and high-grade B-cell non-Hodgkin's lymphoma. Türk Patoloji Derg. 2011;27(3):204–9.
24. Folkman J. Tumor angiogenesis. Adv Cancer Res. 1985;43:175–203.
25. Folkman J. Tumor angiogenesis: therapeutic implications. N Engl J Med. 1971 Nov 18;285(21):1182–6.
26. Folkman J, Klagsbrun M. Angiogenic factors. Science. 1987 Jan 23;235(4787):442–7.
27. Ganjoo K. Antiangiogenesis: a new approach to the treatment of lymphoma. Leuk Lymphoma. 2007 Mar;48(3):454–5.
28. Jørgensen JM, Sørensen FB, Bendix K, Nielsen JL, Olsen ML, Funder AMD, et al. Angiogenesis in non-Hodgkin's lymphoma: clinico-pathological correlations and prognostic significance in specific subtypes. Leuk Lymphoma. 2007 Mar;48(3):584–95.
29. Auerbach W, Auerbach R. Angiogenesis inhibition: a review. Pharmacol Ther. 1994 Sep;63(3):265–311.
30. Cockerill GW, Gamble JR, Vadas MA. Angiogenesis: models and modulators. Int Rev Cytol. 1995;159:113–60.
31. Ferrara N. Role of vascular endothelial growth factor in physiologic and pathologic angiogenesis: therapeutic implications. Semin Oncol. 2002 Dec;29(6 Suppl 16):10–4.

32. Tzankov A, Heiss S, Ebner S, Sterlacci W, Schaefer G, Augustin F, et al. Angiogenesis in nodal B cell lymphomas: a high throughput study. J Clin Pathol. 2007 May;60(5):476–82.
33. Folkman J, D'Amore PA. Blood vessel formation: what is its molecular basis? Cell. 1996 Dec 27;87(7):1153–5.
34. Weidner N. Intratumor microvessel density as a prognostic factor in cancer. Am J Pathol. 1995 Jul;147(1):9–19.
35. Bellamy WT, Richter L, Frutiger Y, Grogan TM. Expression of vascular endothelial growth factor and its receptors in hematopoietic malignancies. Cancer Res. 1999 Feb 1;59(3):728–33.
36. Hazar B, Paydas S, Zorludemir S, Sahin B, Tuncer I. Prognostic significance of microvessel density and vascular endothelial growth factor (VEGF) expression in non-Hodgkin's lymphoma. Leuk Lymphoma. 2003 Dec;44(12):2089–93.
37. Koster A, Raemaekers JMM. Angiogenesis in malignant lymphoma. Curr Opin Oncol. 2005 Nov;17(6):611–6.
38. Podar K, Anderson KC. The pathophysiologic role of VEGF in hematologic malignancies: therapeutic implications. Blood. 2005 Feb 15;105(4):1383–95.
39. Spannuth WA, Sood AK, Coleman RL. Angiogenesis as a strategic target for ovarian cancer therapy. Nat Clin Pract Oncol. 2008 Apr;5(4):194–204.
40. Cursiefen C, Chen L, Borges LP, Jackson D, Cao J, Radziejewski C, et al. VEGF-A stimulates lymphangiogenesis and hemangiogenesis in inflammatory neovascularization via macrophage recruitment. J Clin Invest. 2004 Apr 1;113(7):1040–50.
41. Carmeliet P. Angiogenesis in health and disease. Nat Med. 2003 Jun;9(6):653–60.
42. Veikkola T, Alitalo K. VEGFs, receptors and angiogenesis. Semin Cancer Biol. 1999 Jun;9(3):211–20.
43. Geng L, Chaudhuri A, Talmon G, Wisecarver JL, Wang J. TGF-Beta suppresses VEGFA-mediated angiogenesis in colon cancer metastasis. PloS One. 2013;8(3).

44. Gately S, Li WW. Multiple roles of COX-2 in tumor angiogenesis: a target for antiangiogenic therapy. Semin Oncol. 2004 Apr;31(2 Suppl 7):2–11.
45. Höper MM, Voelkel NF, Bates TO, Allard JD, Horan M, Shepherd D, et al. Prostaglandins Induce Vascular Endothelial Growth Factor in a Human Monocytic Cell Line and Rat Lungs via cAMP. Am J Respir Cell Mol Biol. 1997 Dec 1;17(6):748–56.
46. Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. Nature. 2000 Sep 14;407(6801):249–57.
47. Macchiarini P, Fontanini G, Hardin MJ, Squartini F, Angeletti CA. Relation of neovascularisation to metastasis of non-small-cell lung cancer. Lancet Lond Engl. 1992 Jul 18;340(8812):145–6.
48. Weidner N, Carroll PR, Flax J, Blumenfeld W, Folkman J. Tumor angiogenesis correlates with metastasis in invasive prostate carcinoma. Am J Pathol. 1993 Aug;143(2):401–9.
49. Maeda K, Chung YS, Ogawa Y, Takatsuka S, Kang SM, Ogawa M, et al. Prognostic value of vascular endothelial growth factor expression in gastric carcinoma. Cancer. 1996 Mar 1;77(5):858–63.
50. Toi M, Inada K, Suzuki H, Tominaga T. Tumor angiogenesis in breast cancer: its importance as a prognostic indicator and the association with vascular endothelial growth factor expression. Breast Cancer Res Treat. 1995;36(2):193–204.
51. Hansen S, Sørensen FB, Vach W, Grabau DA, Bak M, Rose C. Microvessel density compared with the Chalkley count in a prognostic study of angiogenesis in breast cancer patients. Histopathology. 2004 May;44(5):428–36.
52. Hansen S, Grabau DA, Sørensen FB, Bak M, Vach W, Rose C. Vascular grading of angiogenesis: prognostic significance in breast cancer. Br J Cancer. 2000 Jan;82(2):339–47.
53. Ruan J, Hyjek E, Kermani P, Christos PJ, Hooper AT, Coleman M, et al. Magnitude of stromal hemangiogenesis correlates with histologic subtype of non-Hodgkin's lymphoma. Clin Cancer Res Off J Am Assoc Cancer Res. 2006 Oct 1;12(19):5622–31.

54. Foss HD, Araujo I, Demel G, Klotzbach H, Hummel M, Stein H. Expression of vascular endothelial growth factor in lymphomas and Castleman's disease. J Pathol. 1997 Sep;183(1):44–50.
55. Gratzinger D, Zhao S, Tibshirani RJ, Hsi ED, Hans CP, Pohlman B, et al. Prognostic significance of VEGF, VEGF receptors, and microvessel density in diffuse large B cell lymphoma treated with anthracycline-based chemotherapy. Lab Investig J Tech Methods Pathol. 2008 Jan;88(1):38–47.
56. Doussis-Anagnostopoulou IA, Remadi S, Turley H, Gindre P, Comley M, Borisch B, et al. Platelet-derived endothelial cell growth factor/thymidine phosphorylase immunohistochemical expression in lymphoid tissue and lymphoid malignancies. Hum Pathol. 1997 Oct;28(10):1146–51.
57. Ribatti D, Vacca A, Nico B, Fanelli M, Roncali L, Dammacco F. Angiogenesis spectrum in the stroma of B-cell non-Hodgkin's lymphomas. An immunohistochemical and ultrastructural study. Eur J Haematol. 1996 Feb;56(1-2):45–53.
58. Vacca A, Ribatti D, Ruco L, Giacchetta F, Nico B, Quondamatteo F, et al. Angiogenesis extent and macrophage density increase simultaneously with pathological progression in B-cell non-Hodgkin's lymphomas. Br J Cancer. 1999 Feb;79(5-6):965–70.
59. Ridell B, Norrby K. Intratumoral microvascular density in malignant lymphomas of B-cell origin. APMIS Acta Pathol Microbiol Immunol Scand. 2001 Jan;109(1):66–72.
60. Arias V, Soares FA. Vascular density (tumor angiogenesis) in non-Hodgkin's lymphomas and florid follicular hyperplasia: a morphometric study. Leuk Lymphoma. 2000 Dec;40(1-2):157–66.
61. Passalidou E, Stewart M, Trivella M, Steers G, Pillai G, Dogan A, et al. Vascular patterns in reactive lymphoid tissue and in non-Hodgkin's lymphoma. Br J Cancer. 2003 Feb 24;88(4):553–9.
62. Passam FH, Alexandrakis MG, Kafousi M, Fotinou M, Darivianaki K, Tsirakis G, et al. Histological expression of angiogenic factors: VEGF, PDGFRalpha, and HIF-1alpha in Hodgkin lymphoma. Pathol Res Pract. 2009;205(1):11–20.

63. Weidner N. Current pathologic methods for measuring intratumoral microvessel density within breast carcinoma and other solid tumors. Breast Cancer Res Treat. 1995;36(2):169–80.
64. Weidner N, Semple JP, Welch WR, Folkman J. Tumor angiogenesis and metastasis--correlation in invasive breast carcinoma. N Engl J Med. 1991 Jan 3;324(1):1–8.
65. Duff SE, Jeziorska M, Kumar S, Haboubi N, Sherlock D, O'Dwyer ST, et al. Lymphatic vessel density, microvessel density and lymphangiogenic growth factor expression in colorectal cancer. Colorectal Dis. 2007 Nov;9(9):793-800.
66. Marinaccio C, Nico B, Maiorano E, Specchia G, Ribatti D. Insights in Hodgkin Lymphoma angiogenesis. Leuk Res. 2014 Aug;38(8):857–61.
67. Ribatti D, Nico B, Ranieri G, Specchia G, Vacca A. The role of angiogenesis in human non-Hodgkin lymphomas. Neoplasia N Y N. 2013 Mar;15(3):231–8.
68. Reilly JT, Nash JR, Mackie MJ, McVerry BA. Distribution of fibronectin and laminin in normal and pathological lymphoid tissue. J Clin Pathol. 1985 Aug;38(8):849–54.
69. Gratzinger D, Zhao S, Marinelli RJ, Kapp AV, Tibshirani RJ, Hammer AS, et al. Microvessel density and expression of vascular endothelial growth factor and its receptors in diffuse large B-cell lymphoma subtypes. Am J Pathol. 2007 Apr;170(4):1362–9.
70. Koster A, van Krieken JHJM, Mackenzie MA, Schraders M, Borm GF, van der Laak JAWM, et al. Increased vascularization predicts favorable outcome in follicular lymphoma. Clin Cancer Res Off J Am Assoc Cancer Res. 2005 Jan 1;11(1):154–61.
71. Vacca A, Ribatti D. Bone marrow angiogenesis in multiple myeloma. Leukemia. 2005 Dec 15;20(2):193–9.
72. Gadducci A, Viacava P, Cosio S, Fanelli G, Fanucchi A, Cecchetti D, et al. Intratumoral microvessel density, response to chemotherapy and clinical outcome of patients with advanced ovarian carcinoma. Anticancer Res. 2003 Feb;23(1B):549–56.

73. Obermair A, Wasicky R, Kaider A, Preyer O, Lösch A, Leodolter S, et al. Prognostic significance of tumor angiogenesis in epithelial ovarian cancer. Cancer Lett. 1999 Apr 26;138(1-2):175–82.
74. Ogawa S, Kaku T, Kobayashi H, Hirakawa T, Ohishi Y, Kinukawa N, et al. Prognostic significance of microvessel density, vascular cuffing and vascular endothelial growth factor expression in ovarian carcinoma: a special review for clear cell adenocarcinoma. Cancer Lett. 2002 Feb 8;176(1):111–8.
75. Terai Y, Ueda M, Kumagai K, Ueki K, Ueki M. Tumor angiogenesis and thymidine phosphorylase expression in ovarian carcinomas including serous surface papillary adenocarcinoma of the peritoneum. Int J Gynecol Pathol Off J Int Soc Gynecol Pathol. 2000 Oct;19(4):354–60.
76. Offersen BV, Knap MM, Marcussen N, Horsman MR, Hamilton-Dutoit S, Overgaard J. Intense inflammation in bladder carcinoma is associated with angiogenesis and indicates good prognosis. Br J Cancer. 2002 Dec 2;87(12):1422–30.
77. Höckel M, Vaupel P. Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. J Natl Cancer Inst. 2001 Feb 21;93(4):266–76.
78. Vaupel P, Mayer A, Höckel M. Tumor hypoxia and malignant progression. Methods Enzymol. 2004;381:335–54.
79. Giaccia AJ. Hypoxic Stress Proteins: Survival of the Fittest. Semin Radiat Oncol. 1996 Jan;6(1):46–58.
80. Brown JM. Tumor hypoxia in cancer therapy. Methods Enzymol. 2007;435:297–321.
81. Petrulio CA, Kim-Schulze S, Kaufman HL. The tumour microenvironment and implications for cancer immunotherapy. Expert Opin Biol Ther. 2006 Jul;6(7):671–84.
82. Chouaib S, Messai Y, Couve S, Escudier B, Hasmim M, Noman MZ. Hypoxia promotes tumor growth in linking angiogenesis to immune escape. Cancer Immun Immunother. 2012;3:21.

83. Muz B, de la Puente P, Azab F, Luderer M, Azab AK. The role of hypoxia and exploitation of the hypoxic environment in hematologic malignancies. Mol Cancer Res. 2014 Oct;12(10):1347–54.
84. Höckel M, Schlenger K, Höckel S, Vaupel P. Hypoxic cervical cancers with low apoptotic index are highly aggressive. Cancer Res. 1999 Sep 15;59(18):4525–8.
85. Harris AL. Hypoxia--a key regulatory factor in tumour growth. Nat Rev Cancer. 2002 Jan;2(1):38–47.
86. Yang Y, Sun M, Wang L, Jiao B. HIFs, angiogenesis, and cancer. J Cell Biochem. 2013 May;114(5):967–74.
87. Rademakers SE, Span PN, Kaanders JHAM, Sweep FCGJ, van der Kogel AJ, Bussink J. Molecular aspects of tumour hypoxia. Mol Oncol. 2008 Jun;2(1):41–53.
88. Santore MT, McClintock DS, Lee VY, Budinger GRS, Chandel NS. Anoxia-induced apoptosis occurs through a mitochondria-dependent pathway in lung epithelial cells. Am J Physiol Lung Cell Mol Physiol. 2002 Apr;282(4):L727–34.
89. Cuvier C, Jang A, Hill RP. Exposure to hypoxia, glucose starvation and acidosis: effect on invasive capacity of murine tumor cells and correlation with cathepsin (L + B) secretion. Clin Exp Metastasis. 1997 Jan;15(1):19–25.
90. Graham CH, Forsdike J, Fitzgerald CJ, Macdonald-Goodfellow S. Hypoxia-mediated stimulation of carcinoma cell invasiveness via upregulation of urokinase receptor expression. Int J Cancer. 1999 Feb 9;80(4):617–23.
91. Hockel M, Schlenger K, Aral B, Mitze M, Schaffer U, Vaupel P. Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix. Cancer Res. 1996 Oct 1;56(19):4509–15.
92. Brizel DM, Scully SP, Harrelson JM, Layfield LJ, Dodge RK, Charles HC, et al. Radiation therapy and hyperthermia improve the oxygenation of human soft tissue sarcomas. Cancer Res. 1996 Dec 1;56(23):5347–50.
93. Sundfør K, Lyng H, Rofstad EK. Oxygen tension and vascular density in adenocarcinoma and squamous cell carcinoma of the uterine cervix. Acta Oncol Stockh Swed. 1998;37(7-8):665–70.

94. Moritz W, Meier F, Stroka DM, Giuliani M, Kugelmeier P, Nett PC, et al. Apoptosis in hypoxic human pancreatic islets correlates with HIF-1alpha expression. FASEB J. 2002 May;16(7):745–7.
95. Akakura N, Kobayashi M, Horiuchi I, Suzuki A, Wang J, Chen J, et al. Constitutive expression of hypoxia-inducible factor-1alpha renders pancreatic cancer cells resistant to apoptosis induced by hypoxia and nutrient deprivation. Cancer Res. 2001 Sep 1;61(17):6548–54.
96. Ryan HE, Lo J, Johnson RS. HIF-1 alpha is required for solid tumor formation and embryonic vascularization. EMBO J. 1998 Jun 1;17(11):3005–15.
97. Jiang BH, Agani F, Passaniti A, Semenza GL. V-SRC induces expression of hypoxia-inducible factor 1 (HIF-1) and transcription of genes encoding vascular endothelial growth factor and enolase 1: involvement of HIF-1 in tumor progression. Cancer Res. 1997 Dec 1;57(23):5328–35.
98. Halterman MW, Miller CC, Federoff HJ. Hypoxia-inducible factor-1alpha mediates hypoxia-induced delayed neuronal death that involves p53. J Neurosci. 1999 Aug 15;19(16):6818–24.
99. Muz B, de la Puente P, Azab F, Azab AK. The role of hypoxia in cancer progression, angiogenesis, metastasis, and resistance to therapy. Hypoxia. 2015 Dec;83.
100. Lu X, Kang Y. Hypoxia and hypoxia-inducible factors: master regulators of metastasis. Clin Cancer Res. 2010 Dec 15;16(24):5928–35.
101. Strese S, Fryknäs M, Larsson R, Gullbo J. Effects of hypoxia on human cancer cell line chemosensitivity. BMC Cancer. 2013 Jul 5;13:331.
102. Chen Y, Zhang L, Pan Y, Ren X, Hao Q. Over-expression of semaphorin4D, hypoxia-inducible factor-1α and vascular endothelial growth factor is related to poor prognosis in ovarian epithelial cancer. Int J Mol Sci. 2012;13(10):13264–74.
103. Hickey MM, Simon MC. Regulation of angiogenesis by hypoxia and hypoxia-inducible factors. Curr Top Dev Biol. 2006;76:217–57.

104. Zhong H, De Marzo AM, Laughner E, Lim M, Hilton DA, Zagzag D, et al. Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. Cancer Res. 1999 Nov 15;59(22):5830–5.

105. Evens AM, Schumacker PT, Helenowski IB, Singh ATK, Dokic D, Keswani A, et al. Hypoxia inducible factor-alpha activation in lymphoma and relationship to the thioredoxin family. Br J Haematol. 2008 May;141(5):676–80.

106. Ganjoo KN, Moore AM, Orazi A, Sen JA, Johnson CS, An CS. The importance of angiogenesis markers in the outcome of patients with diffuse large B cell lymphoma: a retrospective study of 97 patients. J Cancer Res Clin Oncol. 2008 Mar;134(3):381–7.

107. Bhalla S, Evens AM, Prachand S, Schumacker PT, Gordon LI. Paradoxical regulation of hypoxia inducible factor-1α (HIF-1α) by histone deacetylase inhibitor in diffuse large B-cell lymphoma. PloS One. 2013;8(11):e81333.

108. Sadri N, Zhang PJ. Hypoxia-Inducible Factors: Mediators of Cancer Progression; Prognostic and Therapeutic Targets in Soft Tissue Sarcomas. Cancers. 2013 Apr 2;5(2):320–33.

109. Keith B, Johnson RS, Simon MC. HIF1α and HIF2α: sibling rivalry in hypoxic tumour growth and progression. Nat Rev Cancer. 2012 Jan;12(1):9–22.

110. Evens AM, Sehn LH, Farinha P, Nelson BP, Raji A, Lu Y, et al. Hypoxia-inducible factor-1 {alpha} expression predicts superior survival in patients with diffuse large B-cell lymphoma treated with R-CHOP. J Clin Oncol. 2010 Feb 20;28(6):1017–24.

111. Krock BL, Skuli N, Simon MC. Hypoxia-Induced Angiogenesis. Genes Cancer. 2011 Dec;2(12):1117–33.

112. Rankin EB, Rha J, Unger TL, Wu CH, Shutt HP, Johnson RS, et al. Hypoxia-Inducible Factor (HIF)-2 regulates Vascular Tumorigenesis in Mice. Oncogene. 2008 Sep 11;27(40):5354–8.

113. Madanecki P, Kapoor N, Bebok Z, Ochocka R, Collawn JF, Bartoszewski R. Regulation of angiogenesis by hypoxia: the role of micro-RNA. Cell Mol Biol Lett. 2013 Mar;18(1):47-57.

114. Liao D, Johnson RS. Hypoxia: a key regulator of angiogenesis in cancer. Cancer Metastasis Rev. 2007 Jun;26(2):281–90.

115. Pugh CW, Ratcliffe PJ. Regulation of angiogenesis by hypoxia: role of the HIF system. Nat Med. 2003 Jun;9(6):677–84.

116. Zhang J, Grubor V, Love CL, Banerjee A, Richards KL, Mieczkowski PA, et al. Genetic heterogeneity of diffuse large B-cell lymphoma. Proc Natl Acad Sci U S A. 2013 Jan 22;110(4):1398–403.

117. Cardesa-Salzmann TM, Colomo L, Gutierrez G, Chan WC, Weisenburger D, Climent F, et al. High microvessel density determines a poor outcome in patients with diffuse large B-cell lymphoma treated with rituximab plus chemotherapy. Haematologica. 2011 Jul;96(7):996–1001.

118. Nishida N, Yano H, Nishida T, Kamura T, Kojiro M. Angiogenesis in Cancer. Vasc Health Risk Manag. 2006 Sep;2(3):213–9.

119. Tímár J, Döme B, Fazekas K, Janovics A, Paku S. Angiogenesis-dependent diseases and angiogenesis therapy. Pathol Oncol Res POR. 2001;7(2):85–94.

120. Moehler TM, Ho AD, Goldschmidt H, Barlogie B. Angiogenesis in hematologic malignancies. Crit Rev Oncol Hematol. 2003 Mar;45(3):227–44.

121. Ribatti D. Judah Folkman, a pioneer in the study of angiogenesis. Angiogenesis. 2008 Mar;11(1):3–10.

122. Moeller BJ, Cao Y, Vujaskovic Z, Li CY, Haroon ZA, Dewhirst MW. The relationship between hypoxia and angiogenesis. Semin Radiat Oncol. 2004 Jul;14(3):215–21.

123. Zhao W-L, Mourah S, Mounier N, Leboeuf C, Daneshpouy ME, Legrès L, et al. Vascular endothelial growth factor-A is expressed both on lymphoma cells and endothelial cells in angioimmunoblastic T-cell lymphoma and related to lymphoma progression. Lab Invest. 2004 Aug 16;84(11):1512–9.

124. Zhao J, Du F, Shen G, Zheng F, Xu B. The role of hypoxia-inducible factor-2 in digestive system cancers. Cell Death Dis. 2015 Jan;6(1):e1600.

125. Partridge SE, Aquino-Parsons C, Luo C, Green A, Olive PL. A pilot study comparing intratumoral oxygenation using the comet assay following 2.5% and

5% carbogen and 100% oxygen. Int J Radiat Oncol Biol Phys. 2001 Feb 1;49(2):575–80.

126. Durand RE, Raleigh JA. Identification of nonproliferating but viable hypoxic tumor cells in vivo. Cancer Res. 1998 Aug 15;58(16):3547–50.

127. Farinha P, Kyle AH, Minchinton AI, Connors JM, Karsan A, Gascoyne RD. Vascularization predicts overall survival and risk of transformation in follicular lymphoma. Haematologica. 2010 Dec;95(12):2157–60.

128. Ribatti D, Vacca A, Marzullo A, Nico B, Ria R, Roncali L, et al. Angiogenesis and mast cell density with tryptase activity increase simultaneously with pathological progression in B-cell non-Hodgkin's lymphomas. Int J Cancer. 2000 Jan 15;85(2):171–5.

129. Wobser M, Siedel C, Kneitz H, Bröcker E-B, Goebeler M, Houben R, et al. Microvessel density and expression of vascular endothelial growth factor and its receptors in different subtypes of primary cutaneous B-cell lymphoma. Acta Derm Venereol. 2013 Nov;93(6):656–62.

130. Alizadeh AA, Eisen MB, Davis RE, Ma C, Lossos IS, Rosenwald A, et al. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. Nature. 2000 Feb 3;403(6769):503–11.

131. Lossos IS, Czerwinski DK, Alizadeh AA, Wechser MA, Tibshirani R, Botstein D, et al. Prediction of survival in diffuse large-B-cell lymphoma based on the expression of six genes. N Engl J Med. 2004 Apr 29;350(18):1828–37.

132. Lossos IS, Morgensztern D. Prognostic biomarkers in diffuse large B-cell lymphoma. J Clin Oncol. 2006 Feb 20;24(6):995–1007.

133. Kim MK, Suh C, Chi HS, Cho HS, Bae YK, Lee KH, et al. VEGFA and VEGFR2 genetic polymorphisms and survival in patients with diffuse large B cell lymphoma. Cancer Sci. 2012 Mar 1;103(3):497–503.

134. Dou H, Hu J, Tang Y, Lin W. Clinical significances of serum levels of VEGF and the relationship with IPI in the patients with diffuse large B-cell lymphoma. J Leuk Lymphoma. 2009;155–7.

135. Powell JR, Dojcinov S, King L, Wosniak S, Gerry S, Casbard A, et al. Prognostic significance of hypoxia inducible factor-1α and vascular endothelial

growth factor expression in patients with diffuse large B-cell lymphoma treated with rituximab. Leuk Lymphoma. 2013 May 1;54(5):959–66.

136. Mucci LA, Powolny A, Giovannucci E, Liao Z, Kenfield SA, Shen R, et al. Prospective Study of Prostate Tumor Angiogenesis and Cancer-Specific Mortality in the Health Professionals Follow-Up Study. J Clin Oncol. 2009 Nov 20;27(33):5627–33.

137. de Mendonça GRA, Brito ABC, Rocha RM, Delamain MT, de Andrade Natal R, Soares FA, et al. Association of VEGFA-2578 C>A polymorphism with clinicopathological aspects and outcome in follicular lymphoma patients. Blood Cancer J. 2016 Aug;6(8):e464.

138. Lozano-Santos C, Martinez-Velasquez J, Fernandez-Cuevas B, Polo N, Navarro B, Millan I, et al. Vascular Endothelial Growth Factor A (VEGFA) Gene Polymorphisms Have an Impact on Survival in a Subgroup of Indolent Patients with Chronic Lymphocytic Leukemia. PLoS ONE. 2014 Jun 27(6):e101063.

139. Aguayo A, Kantarjian H, Manshouri T, Gidel C, Estey E, Thomas D, et al. Angiogenesis in acute and chronic leukemias and myelodysplastic syndromes. Blood. 2000 Sep 15;96(6):2240–5.

140. Relander T, Johnson NA, Farinha P, Connors JM, Sehn LH, Gascoyne RD. Prognostic factors in follicular lymphoma. J Clin Oncol. 2010 Jun 10;28(17):2902–13.

141. Pagé EL, Robitaille GA, Pouysségur J, Richard DE. Induction of hypoxia-inducible factor-1alpha by transcriptional and translational mechanisms. J Biol Chem. 2002 Dec 13;277(50):48403–9.

142. Azab AK, Hu J, Quang P, Azab F, Pitsillides C, Awwad R, et al. Hypoxia promotes dissemination of multiple myeloma through acquisition of epithelial to mesenchymal transition-like features. Blood. 2012 Jun 14;119(24):5782–94.

143. Wouters BG, Koritzinsky M. Hypoxia signalling through mTOR and the unfolded protein response in cancer. Nat Rev Cancer. 2008 Nov;8(11):851–64.

144. Semenza GL, Wang GL. A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. Mol Cell Biol. 1992 Dec;12(12):5447–54.

145. Stewart M, Talks K, Leek R, Turley H, Pezzella F, Harris A, et al. Expression of angiogenic factors and hypoxia inducible factors HIF 1, HIF 2 and CA IX in non-Hodgkin's lymphoma. Histopathology. 2002 Mar;40(3):253–60.

146. Talks KL, Turley H, Gatter KC, Maxwell PH, Pugh CW, Ratcliffe PJ, et al. The expression and distribution of the hypoxia-inducible factors HIF-1alpha and HIF-2alpha in normal human tissues, cancers, and tumor-associated macrophages. Am J Pathol. 2000 Aug;157(2):411–21.

147. Semenza GL. Expression of hypoxia-inducible factor 1: mechanisms and consequences. Biochem Pharmacol. 2000 Jan 1;59(1):47–53.

148. Taskinen M, Jantunen E, Kosma V-M, Bono P, Karjalainen-Lindsberg M-L, Leppä S. Prognostic impact of CD31-positive microvessel density in follicular lymphoma patients treated with immunochemotherapy. Eur J Cancer Oxf Engl 1990. 2010 Sep;46(13):2506–12.

11. CURRICULUM VITAE

Labinot Shahini was born on June 27 1981. in Pristina, Republic of Kosovo, where he graduated from the Faculty of Medicine, University of Pristina in 2006. Since 2007 he works as a teaching assistant at Faculty of Medicine, University of Pristina.

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He participated in many congresses, conferences, and seminars, in Kosovo, and many other countries.

He is married and has a daughter and a son.