

**UNIVERSITY OF MEDICINE AND PHARMACY OF CRAIOVA
DOCTORAL SCHOOL**



THESIS SUMMARY

**DIAGNOSTIC, HYSTOPATHOLOGICAL AND
IMMUNOHISTOCHEMICAL ASPECTS IN DIFFUSE LARGE B CELL
LYMPHOMA**

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STATE OF KNOWLEDGE

I. LYMPHOID TISSUE AND LYMPHOPOIETIC ORGANS

The lymphoid tissue and the lymphopoietic organs are the two essential components of the lymphatic system. The lymphoid tissue is composed of diffuse lymphoid tissue and lymphatic follicles. The lymphopoietic organs are represented by the thymus, the spleen, and the homomedulla, during the intrauterine life.

II. ETIOPATHOGENY OF DIFFUSE LARGE B CELL LYMPHOMA

NHLs account for roughly 85% of all lymphomas, and are most commonly classified as B-cell, T-cell and NK (natural killer) cell lymphoma, according to their cell of origin. Diffuse large B-cell lymphoma is a special type of aggressive lymphoma and represent the most common subtype of NHL. A series of genetic abnormalities including numerical alterations, point mutations, and, more rarely, translocations and gene amplifications are involved in the pathogenesis of this type of lymphoma and may be associated with certain histological and immunophenotypic variants.

III. CLASSIFICATION AND STADIALIZATION

A number of different classifications for non-Hodgkin lymphomas have been suggested during the last half-century, each with its own advantages and drawbacks: *Rappaport*, *Kiel*, *International Working Formulation (IWF)*, *REAL* (Revised European-American Lymphoma classification). The last classification, that is currently in use, is the one offered by the WHO in 2001, last revised in 2016. According to the maturation stage in which the B cell is, and to the type of anomalies that occur during differentiation and maturation, DLBCL presents several variants and subtypes.

IV. MORPHOLOGY AND CYTOGENETICS

The Hans algorithm structured DLBCL in two main subtypes of the germinal center (GCB) and non-germinal center (non-GCB) by analyzing three essential markers: CD10, MUM1, and bcl-6. The same classification can be achieved by using GCTE1, CD10, BCL-6, MUM1 and FOXP1 biomarkers (the Tally algorithm) with a slightly better accuracy. However, the detection of other

cell markers expression has become a fundamental component both in establishing an accurate prognosis, and in the development of an optimal therapeutic algorithm.

V. DIAGNOSIS

DLBCL patients have a wide spectrum of clinical manifestations, a frequent nodal involvement, but also, in up to 40% of cases, extranodal symptoms (skin, digestive, central nervous system etc). All these clinical manifestations, together with the general signs and symptoms, usually indicate a more aggressive phenotype. As for the paraclinical investigations, usual blood tests consist in complete blood count, lactate dehydrogenase and uric acid determination, serology, osteomedular biopsy and cytologic exam of the cerebrospinal fluid. CT scan or, even better, PET-CT are considered mandatory to assess the extension of the disease.

VI. TREATMENT OF DIFFUSE LARGE B CELL LYMPHOMA

The treatment protocol in DLBCL did not change much during the last twenty years. One notable exception is the introduction of Rituximab as an important addition to standard chemotherapy protocol in CD20+ cases, providing significant improvement in complete remission rate, disease-free survival and overall survival, with minimal added toxicity. Nevertheless, up to 15% of patients diagnosed with DLBCL exhibit primary refractory disease and 20-25% relapse after the initial response to R-CHOP regimen. Therefore, other therapeutic solutions have been tested, such as R-miniCHOP, R-ACVBP, R-CHOEP etc. All these regimes are strictly codified by several therapeutic protocols, such as the 2015 ESMO guidelines.

VII. STEMM CELL TRANSPLANTATION

Autologous and allogeneic hematopoietic stem-cell transplantation (allo-SCT) can provide viable therapeutic options for certain cases of non-Hodgkin lymphoma, such as relapsed, advanced and otherwise incurable NHL. Different scoring systems have been proposed in the last decades to better select the candidates for transplant. The hematopoietic stem cell transplantation-specific comorbidity index has been established as an important tool to assess the risk of non-relapse mortality following allo-SCT. This index does not take into account either the disease stage at transplant or the donor type, in contrast to the EBMT index.

ORIGINAL STUDY

I. MOTIVATION AND STUDY PREMISES

The aim of this study was to identify the clinicopathological factors that correlate with an unfavorable prognosis in DLBCL patients, as well as to assess their potential therapeutic implications.

This work also aimed to assess the prognostic value of four IHC markers, other than those used in Hans protocol, by investigating the correlation between their expression and International prognostic index (IPI) score, as well as by comparing two important survival endpoints (disease-free survival and overall survival).

The third objective of this study was to evaluate three Rituximab-based treatment regimens in DLBCL according to their short-term biological impact, overall and disease-free survival rate.

The protocol of this study was in concordance with the Romanian and European legislation, and had the approval of the Research and Ethics Committee of the University of Medicine and Pharmacy of Craiova.

II. CLINICO-DEMOGRAPHIC STUDY

II.1. Material and methods

We selected 97 patients diagnosed with de novo DLBCL in the Filantropia Hospital of Craiova from January 2007 to December 2016. The diagnosis of DLBCL and its two immunohistochemical subtypes relied on the 2008 WHO criteria. The inclusion parameters consisted in the availability of clinical, morphological and therapeutic data for each patient, as well as the immunohistochemical confirmation of GCB/non-GCB subtype using Hans algorithm. We excluded cases with a previously indolent lymphoma that suffered subsequent transformation into a DLBCL and immunodeficiency-associated lymphomas. In addition to the clinical and histological data, each patient had complete blood cell count, comprehensive metabolic panel (LDH evaluation, liver and renal function) and bone marrow biopsy performed before the initiation of therapy.

We investigated the prognostic value of IPI score in GCB and non-GCB DLBCL in patients treated with conventional chemotherapy regimens according to ESMO guidelines.

Statistical analysis: The categorical variables (such as clinicopathological factors, histologic classification according to Hans algorithm, IPI score etc.) were assessed using Fisher's exact test. The survival functions for the overall survival (OS), defined as the time between the initiation of treatment until death, and disease-free survival (DFS) were assessed using the Kaplan-Meier method. Log-rank test was applied to conceive a prognostic model for disease-free survival and overall survival. The descriptive statistics were performed using Microsoft Excel Data Analysis module along with XLSTAT suite (Microsoft Corp, USA). All other statistical tests were performed using GraphPad Prism 7.0 (GraphPad Software Inc. San Diego, USA) with $p \leq 0.05$ being considered statistically significant.

II.2. Results

The clinical profile of the patients indicated a M: F sex ratio of 1.32, while mean age (\pm std dev) was 56 (\pm 14.67 years). Among the various clinical pathological features of the DLBCL patients, presence of B symptoms, and positive therapeutic response indicated statistically significant levels of correlation with the IPI score, while gender did not seem to influence the prognostic index. The non-GCB subtype is of particular interest, as it shows a very strong correlation with the IPI scoring system ($p < 0.0001$, OR=11.97, CI 95% :4.527-30.99).

Overall survival was compared in four subgroups: low IPI vs. high IPI patients, and GCB DLBCL vs. non-GCB DLBCL. Patients with a high prognostic index exhibited lower survival rates ($p < 0.001$). Mean survival rate in this subgroup was 23.53 months, while the low IPI patients had a mean survival of 36.87 months.

Statistical significant difference in overall survival rate and disease-free survival rate was also observed when DLBCL subtype distribution was analyzed.

III. HISTOPATHOLOGICAL AND IMMUNOHISTOCHEMICAL STUDY

III.1. Material and methods

For the current study, we analyzed 97 formalin-fixed, paraffin-embedded tissue samples of the DLBCL patients admitted and treated in the Hematology Department of Craiova. The specimens were surgically or endoscopically retrieved for diagnostic confirmation and were preserved in the Pathology Department of the Emergency County Hospital of Craiova.

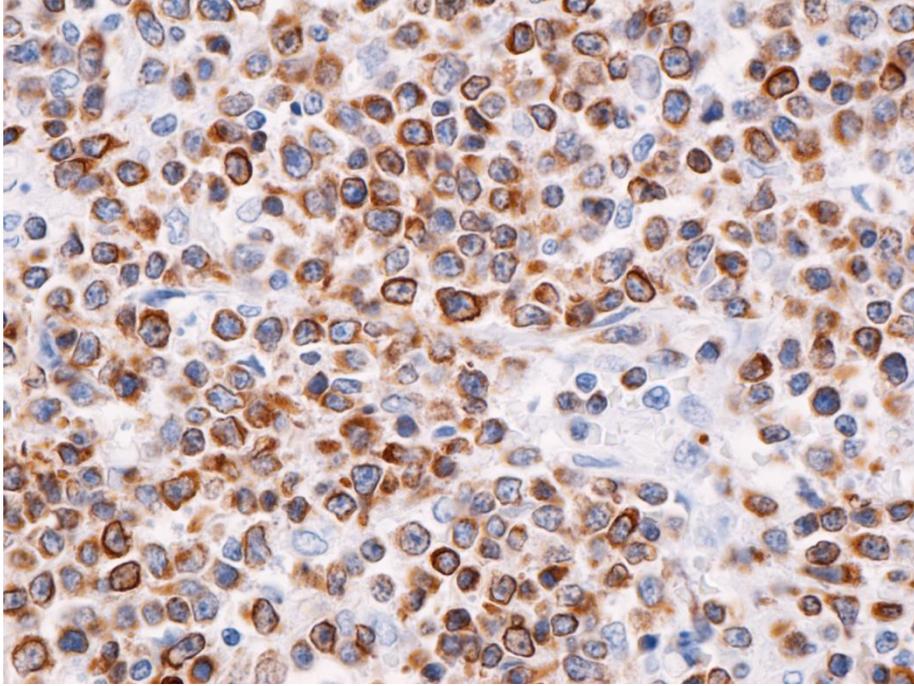
The paraffin-embedded tissue blocks were cut at 4 μm sections. For histological analysis hematoxylin-eosin (HE) and trichromic Goldner-Szeckeli (GS) staining was subsequently performed. For the immunohistochemical assessment seven markers were considered: bcl2, Ki67, p53, and MYC, for prognostic evaluation, and bcl6, MUM1 and CD10, for Hans algorithm. Several other markers were analyzed in a limited number of cases: CD20, CD34, and CD79a. Similar statistical tests were used for correlation and survival assessment.

III.2. Results

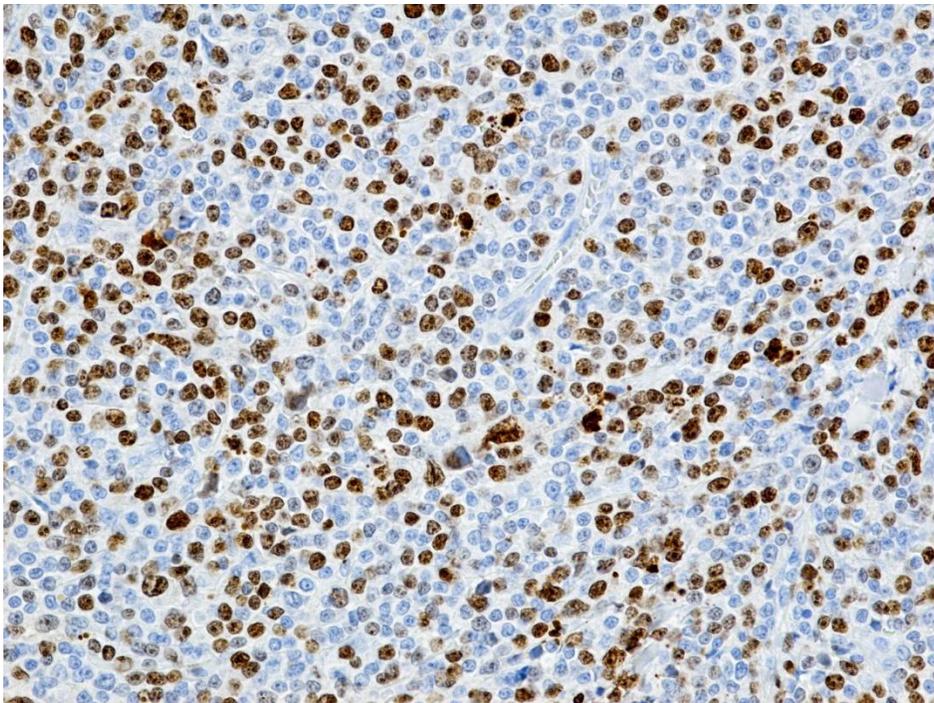
The histological and immunohistochemical study was performed on 97 tissue samples. Histopathological examination using HE and GS stains revealed intense lymphoblastic cell proliferation, with round or oval shaped, hypochromic nuclei containing smooth inhomogeneous chromatin and 2-3 recognizable nucleoli. Sometimes the nuclei displayed vesicular or pleomorphic architecture. The cytoplasm was light acidophilic and poorly represented in centroblastic lymphomas, and basophilic in immunoblastic lymphomas. Cellular proliferation led to the disruption and disorganization of the tissues.

In terms of immunohistochemical reaction, an intense reaction to the anti-bcl2 antibody was observed in about 50% of cases, indicating an overexpression of this marker in tumoral cells. In order to assess the proliferative activity of the tumoral cells, we used the anti-Ki67 antibody, which is the marker for a nuclear non-histone protein synthesized at the beginning of the cellular proliferation cycle. We accounted more than half of the cases with an intense immunohistochemical reaction to anti-Ki67 antibody. In our study, the immunohistochemical reaction for p53 was positive in about 25% of cases, and almost 32% of cases had a positive expression for MYC.

Forty-four cases (45.36%) were germinal center B-cell DLBCL, and 53 (54.64%) were non-germinal center lymphomas. Bcl-2 displayed similar distribution in both subgroups (positivity in 41.67% of cases for GCB and in 58.33% of non-GCB patients, $p > 0.05$). Likewise, no correlations could be observed for p53 expression. However, a strong correlation between MYC positive expression and non-BCL subtype was observed ($p < 0.005$, $OR = 4.374$), with similar results found in Ki67 expression ($p < 0.001$, $OR = 7.382$). When the IPI distribution across the group was investigated, significant correlations were observed between high prognostic index values (> 2) and positive expression for all four markers.



Diffuse large B-cell lymphoma. Intense membrane staining for bcl2 in tumoral lymphocytes;
x400.



Diffuse large B-cell lymphoma. Positive Ki67 expression in 70-80% of tumoral cells nuclei;
x200.

The median survival for the full cohort was 26 months. However, for a more rigorous survival analysis, of the 97 newly diagnosed cases with DLBCL, we selected only the patients subjected to standard R-CHOP treatment. Kaplan-Meier analysis of the 78 patients that followed R-CHOP regimen revealed a tendency toward decreased 5-years overall survival (32.6% vs. 52.4%, $p < 0.001$) and disease-free survival (23.9% vs. 77.5%, $p < 0.001$) in patients with positive Bcl2 expression than for those cases with reduced or negative expression. Similarly, statistically significant differences were also observed when p53 expression was analyzed. Overall survival in p53 negative group was 55.6%, while for p53 expressing cases the 5 years survival was 22.8% ($p = 0.0034$). Furthermore, significant differences were also observed in disease-free survival distribution, with p53 negative patients exhibiting higher DFS proportions (65.7% vs. 35.3%, $p = 0.0175$). The overall survival in MYC+ group was significantly lower (9.72%) than in MYC group (55.2%, $p < 0.001$), with a similar tendency being observed in case of disease free survival.

IV. ASSESSMENT OF THE THERAPEUTIC RESPONSE

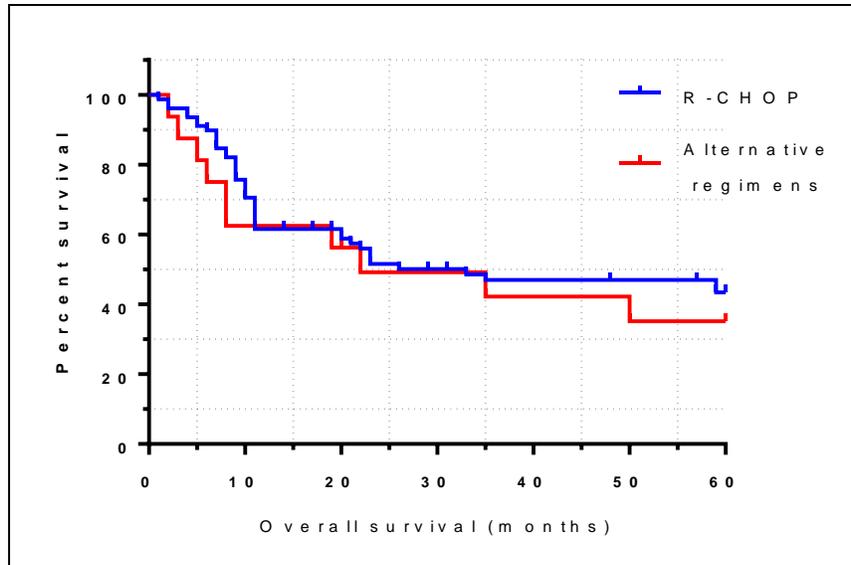
IV.1. Material and methods

One hundred and twenty-eight DLBCL cases were diagnosed and classified according to the 2008 World Health Organization classification of tumors of hematopoietic and lymphoid tissues. However, we selected only those cases with confirmed DLBCL that followed Rituximab-based chemotherapy, and underwent the complete therapeutic protocol. Same statistical tools were used as in the previous two studies.

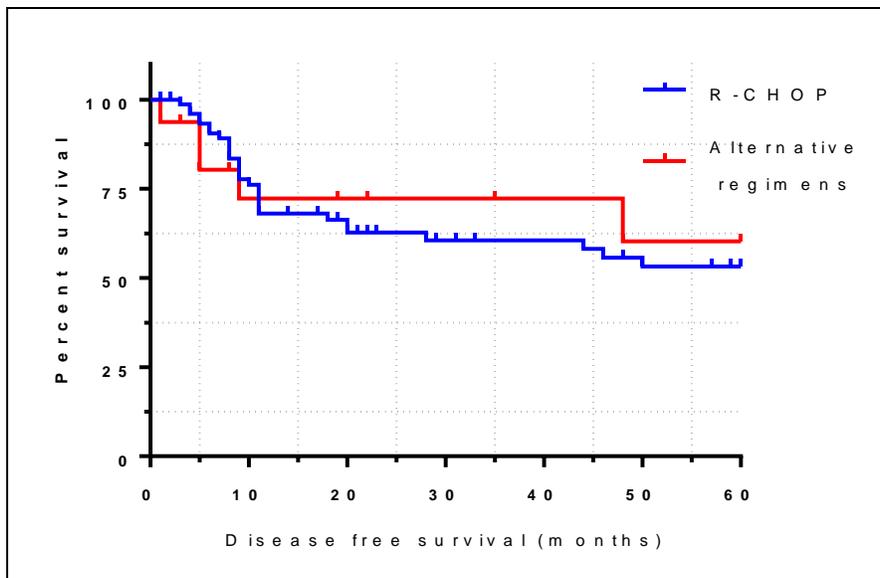
IV.2. Results

Ninety-four patients were selected according to the inclusion criteria. The following regimens were used: R-CHOP, R-CHOEP, and R-miniCHOP. Mean age in the R-CHOP group was 53.2 years, while in the alternative rituximab-based regimen (R-CHOEP and R-miniCHOP) was 66.5 years. The patients were divided in two groups, both including Rituximab as an indispensable therapeutic agent. The first group of 78 cases was treated with R-CHOP regimen, while the second group, consisting of 16 cases, included all patients with other Rituximab-associated regimen, such as R-miniCHOP and R-CHOEP. Relevant blood test analysis indicated variations of their mean values

at different timeframes (T0 – the beginning of therapy, T1 – end of therapy/6 months). However, no statistical difference was observed between the groups.



Overall survival for R-CHOP vs. alternative Rituximab-based regimens in DLBCL patients



Disease-free survival for R-CHOP vs. alternative Rituximab-based regimens in DLBCL patients

The comparative analysis of the Kaplan-Meier curves in the two groups indicated no statistical difference regarding the 5-year overall survival, with an average survival rate of 43.36% in the R-CHOP group, and 35.15% in the second group. When disease-free survival was analyzed similar

results were observed. There was no statistical difference between the R-CHOP and the alternative Rituximab-based regimens. The mean survival rate in the first group was 53.18%, and 60.26% in the second group.

V. DISCUSSIONS

The role of immunophenotype variability for the therapeutic outcome has long been the cornerstone for DLBCL management strategy, largely because the treatment of lymphomas evolves towards targeted therapies, which is achieved by understanding tumor biology and by discovering new signaling pathways. Moreover, several biological therapies are available today, ranging from the already known interferon therapy, to rituximab or radiolabeled antibodies, to name just a few of the recent acquisitions in the DLBCL therapeutic armamentarium. The R-CHOP treatment, which is the main regimen for the CD20+ lymphomas, can provide a favorable therapeutic response in only 40-50% of patients, therefore it is important to establish from the moment of diagnosis if it is necessary to apply a more aggressive or even an experimental therapy. The process through which this can be achieved is by independently assessing the morphologic, immunohistochemical and genetic profile of every patient selected for individualized treatment.

VI. CONCLUSIONS

The routine assessment of DLBCL cell-of-origin classification according to Hans algorithm, and the IPI score are essential, as both predictors positively correlate with multiple clinico-therapeutical parameters, and can serve as a useful instrument in different survival endpoint assessment.

The positive expression of Bcl2, MYC, p53, and Ki-67 positively correlates with low prognostic index and poor survival rate, thus making them valuable diagnostic and therapeutic targets.

No significant differences in treatment endpoints between the two therapy groups were recorded, supporting the hypothesis that all three Rituximab-based regimens (R-CHOP, R-miniCHOP and R-CHOEP) provide good therapeutic alternatives for DLBCL patients with dose-dependent adaptation based on age and comorbidities.

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