UNIVERSITY OF MEDICINE AND PHARMACY
"CAROL DAVILA" BUCHAREST
DOCTORAL SCHOOL
MEDICINE

MORPHOLOGICAL AND IMMUNOPHENOTYPIC VARIABILITY IN HIGH-GRADE B-CELL NON-HODGKIN LYMPHOMAS

ABSTRACT OF THE DOCTORAL THESIS

PhD supervisor:
PROF. UNIV. HABIL. DR. CEAUȘU MIHAIL CONSTANTIN

PhD student:
HALCU GEORGIAN

2025

Table of contents

General part **9**

1. Introduction **10**

2. B lymphocyte **13**

2.1. B lymphocyte - signaling pathways and microenvironment 13

3. Non-Hodgkin's large B-cell lymphoma – updates in classification **16**

3.1. Evolution of the classification in large B-cell non-Hodgkin lymphoma 16

3.2. Diffuse large cell B lymphoma, NOS (DLBCL, NOS) 18

3.3. T-cell/histiocyte-rich large B-cell lymphoma (THR-LBCL) 19

3.4. Diffuse large B-cell lymphoma/ High-grade B-cell lymphoma with MYC and BCL2 rearrangements (DLBCL/HGBL-MYC/BCL2) 20

3.5. ALK-positive large B-cell lymphoma (ALK+ LBCL) 21

3.6. Large B-cell lymphoma with IRF4 rearrangements (IRF4-LBCL) 21

3.7. High-grade B-cell lymphoma with 11q aberrations (HGBL – 11q) 22

3.8. Lymphomatoid granulomatosis (LYG) 22

3.9. EBV positive Diffuse large B-cell lymphoma, (EBV-DLBCL) 23

3.10. Diffuse large B-cell lymphoma associated with chronic inflammation (CI-DLBCL) 24

3.11. Fibrin-associated large B-cell lymphoma (FA-LBCL) 25

3.12. Fluid overload-associated Large B-cell Lymphoma (FO-LBCL) 26

3.13. Plasmablastic lymphoma (PBL) 27

3.14. Large B-cell lymphoma, primary of immunoprotected sites 28

3.15. Primary cutanous Diffuse large B-cell lymphoma, leg type (PCLBCL-LT) 29

3.16. Intravascular large B-cell lymphoma (IV-LBCL) 29

3.17. Primary mediastinal B-cell Lymphoma (PMBCL) 30

3.18. Mediastinal gray zone B-cell lymphoma (MGZL) 31
3.19. High-grade B-cell lymphoma NOS (HGBL NOS) 31
4. Elements of Molecular Biology and Cytogenetics in Non-Hodgkin's Large B-cell Lymphoma 34
4.1. Molecular subtypes of DLBCL 34
4.2. Genetic and cytogenetic alterations in DLBCL 34
4.3. Chromosomal translocations 35
4.4. Genetic mutations 35
4.5. Molecular and cytogenetic investigation techniques 35
4.6. Clinical and therapeutic implications 35
Original part 37
5. Working hypothesis and general objectives 38
6. General research methodology 40
6.1. Sample collection and construction of tissue micro-array (TMA) blocks 41
6.2. Immunohistochemical examination 43
6.3. Statistical analysis 44
7. From biopsy to diagnosis: Large B-cell lymphoma in practice 46
7.1. Introduction 46
7.2. Lymphoma, carcinoma, melanoma or sarcoma – what are the differences? 46
7.3. B-cell, T-cell non-Hodgkin's lymphoma or Hodgkin's lymphoma 48
7.4. Growth pattern and histological elements 49
7.5. Morphological assessment 50
7.6. Medium-sized B-cell proliferations 53
7.7. Germinal center phenotype 54
7.8. Hans algorithm 55
7.9. B-cell lymphomas of the double expressor type 56
7.10. Ki67 Proliferation Index 57

7.11. Discussions 60

8. Evaluation of BCL2, c-MYC, and Ki67 Expression in High-Grade B-Cell Non-Hodgkin Lymphoma 61

8.1. Introduction 61

8.2. Materials and methods 62

8.3. Patients 62

8.4. Microscopic and immunohistochemical examination 63

8.5. Statistical analysis 67

8.6. Results 67

8.7. Characteristics of patients 67

8.8. Overall survival and correlations with biological markers 71

8.9. Discussions 75

9. PDL-1, PD-1 Expression and Micromedium in Aggressive B-Cell Non-Hodgkin Lymphoma 79

9.1. Introduction 79

9.2. Materials and methods 80

9.3. Results 87

9.4. Discussions 92

10. Conclusions and personal contributions 97

10.1. Conclusions 97

10.2. Personal contributions 17

Bibliography 101

I. The fundamental issue

Despite significant advances in oncology and hematology over recent decades, certain types of blood cancers, particularly aggressive B-cell non-Hodgkin lymphomas, remain insufficiently understood, especially at the national level[1]. This pathology, marked by biological aggressiveness and rapid progression, presents significant challenges related to diagnosis, treatment, and prognosis, making it a true public health issue[1].

Globally, non-Hodgkin lymphomas rank as the most prevalent hematologic malignancies among adults, with the high-grade B-cell subtype being the most common and exhibiting a disproportionate impact relative to its prevalence. Although these lymphomas account for merely 3–4% of all cancer cases, they are among the top ten malignancies and significantly contribute to oncological mortality, as evidenced by their ranking as the seventh most prevalent in the United States in terms of incidence and ninth regarding mortality. In Europe, the incidence rate ranges from 3.8 to 5 cases per 100,000 inhabitants annually; however, these statistics are influenced by various demographic and genetic factors, as well as disparities in access to healthcare services.

In Romania, the absence of a functional national registry for non-Hodgkin's lymphomas creates a significant gap in epidemiological information[2]. The reported cases are incomplete, and access to centralized data is lacking. According to Globocan 2020, Romania registered about 1900 new cases of non-Hodgkin lymphomas annually, with almost 800 deaths; however, these figures are likely underestimated. In the absence of reliable data, healthcare planning, research, and prevention strategies are severely affected.

A significant issue is the late diagnosis; most patients present at advanced stages, partly due to insufficient training of doctors in the primary care network and limited access to specialized testing. Additionally, high-grade B lymphomas show considerable morphological and immunophenotypic variability, which greatly complicates histopathological diagnosis and classification. Variations in the expression of the BCL2, MYC, and Ki-67 markers, along with changes in the tumor microenvironment (including PD-1, PD-L1, CD4+ T lymphocytes, CD8+ T lymphocytes, and CD68+ macrophages), affect prognosis and treatment response[3].

In Romania, the availability of advanced molecular biology and cytogenetic tests is limited, which impacts both the current diagnostic activities in laboratories and the research capacity[4, 5]. This results in a diagnosis that is sometimes incomplete or inaccurate, and a lack of effective treatment personalization[6, 7].

The present paper begins with the hypothesis that the morphological and particularly immunophenotypic variability of B-cell non-Hodgkin lymphoma holds significant predictive value regarding clinical evolution and treatment response. This study examines the correlation between histological and immunohistochemical factors (BCL2, MYC, Ki67) and clinical evolution data, aiming to identify distinct subgroups with prognostic significance[8].

A complementary direction is to investigate the relationship between the expression of immune checkpoint markers (PD-L1, PD-1) and the infiltration of non-tumor immune cells (T lymphocytes, macrophages) in the tumor microenvironment to enhance our understanding of the mechanisms of immune evasion and potential therapeutic targets.[9].

The primary goal of the thesis is to generate relevant data in the Romanian context, which will enhance the refinement of histopathological diagnosis, aid in the identification of prognostic markers, and ultimately improve personalized treatment for these patients[10].

This research addresses a significant and urgent need to narrow the knowledge gap in Romanian hemato-oncology. By integrating morphological, immunohistochemical, and clinical analyses, the paper proposes a model that can be applied in current medical practice, with the potential to enhance the rate of early diagnosis, improve classification accuracy, and increase treatment efficiency. Additionally, it contributes to understanding the biological diversity of high-grade B-cell non-Hodgkin lymphomas and supports efforts to align the Romanian medical system with international standards.

II. Working hypothesis and general objectives of the research.

The morphological and immunohistochemical variability of large B-cell non-Hodgkin lymphomas reflects the biological heterogeneity of this entity and significantly correlates with the molecular profile, cell subtype, and clinical behavior of the tumor.[11-16]. A detailed characterization of these aspects can lead to a more accurate classification, assist in the prognostic stratification of patients, and aid in the selection of personalized treatment.

The present thesis aims to investigate the morphological and immunophenotypic variability of these neoplasms, combining clinical-pathological observations with modern analyses to achieve a comprehensive overview of the disease. By approaching a topic that has been insufficiently explored at the local level, we aspire to gather valuable data that will contribute to the development of more accurate diagnostic methods and more effective therapeutic strategies.

We start from the premise that large B-cell non-Hodgkin lymphoma, the most common form of aggressive B-cell non-Hodgkin lymphoma, exhibits considerable morphological, immunophenotypic, and molecular heterogeneity, which profoundly influences the tumor's biological behavior, response to treatment, and patient prognosis. This diversity can be explored and understood through an integrative analysis of classical histopathological features, immunohistochemical expression profiles, and molecular subtyping. Supporting these elements is the first study, which contextualizes the multitude of aggressive non-Hodgkin B-cell lymphoma subtypes according to the recent 2022 classification of hematopoietic and lymphoid tumors by the World Health Organization[17].

In the context of diagnosing and evaluating histoprognostic factors, the main working hypothesis was materialized in the second research study, which focused on assessing the expression of the immunohistochemical markers C-MYC, BCL2, and Ki67, alongside a detailed analysis of tumor cell tissue architecture and cytological features. Thus, we aimed to determine how many valuable clues these elements could provide for the precise classification of the tumor into a specific prognostic category and for guidance toward a personalized therapeutic strategy.[18-21].

Another important point was analyzing the expression of the immunoregulatory molecules PD-1 and PDL-1 in the tumor microenvironment of large B-cell non-Hodgkin

lymphoma. In the third study, we aimed to determine whether the altered expression of the PD-1/PDL-1 axis correlates with the differentiated presence of helper T lymphocytes ($CD4^+$), cytotoxic T lymphocytes ($CD8^+$), and macrophages ($CD68^+$) in the tumor microenvironment. Additionally, we sought to explore whether certain morphological subtypes of large B-cell lymphoma are associated with the expression of these checkpoint molecules. The hypothesis is grounded in the idea that hematological malignancies can employ immune evasion mechanisms by inhibiting lymphocyte function, and that the expression of PD-1 on T lymphocytes and its PDL-1 ligand on tumor cells or other cells in the microenvironment contributes to maintaining a suppressive immune status. Furthermore, the distribution and density of $CD4^+$, $CD8^+$, and $CD68^+$ cells may reflect local immune activity and the degree of tumor inflammation, potentially possessing prognostic value[3, 10, 22-24].

Therefore, characterizing these markers in non-Hodgkin large B-cell lymphoma can provide important insights into the mechanisms of tumor progression, the correlations with treatment response, and new therapeutic opportunities, particularly concerning anti-PD-1/PDL-1 immunotherapy.

We also aimed to evaluate the prognostic significance of the histological and immunohistochemical characteristics identified, in relation to the clinical parameters and the progression of the disease. This could facilitate the development of an integrative diagnostic assessment model based on morphology and immunophenotype, which would support current medical practice in risk stratification and guide personalized treatment for patients with lymphoma.

III. General research methodology

This research is retrospective and involved 66 adult patients diagnosed with large B-cell non-Hodgkin lymphoma, who were treated between 2017 and 2024 at Colțea Clinical Hospital in Bucharest (Pathological Anatomy and Hematology departments). The study was conducted with the approval of the Ethics Committee (no. 8541/15.05.2020).

The data were extracted from the hospital's computer databases (InfoWorld and Hippocrates) and supplemented with clinical information from physical and electronic files. The inclusion criteria were: confirmed diagnosis of DLBCL, comprehensive immunohistochemical investigations, and complete medical records. Patients with other types of specific B lymphoma (e.g., Burkitt, CNS, mediastinal), untreated HIV/AIDS, organ transplantation, solid tumors, or severe comorbidities were excluded.

Initially, 125 cases were identified; however, after applying the exclusion criteria, 66 were retained. For these cases, a database was created that includes clinical, pathological, and immunophenotypic parameters such as Ann-Arbor score, LDH, IPI, and treatment.

Tumor samples (paraffin blocks) were retrieved from the pathological anatomy archive, sectioned, stained with HE, and reviewed for tissue quality. Representative tumor areas were selected while avoiding necrosis and artifacts.

To standardize the analysis, four tissue micro-array blocks were created, each containing two tissue cores (2 mm) from each patient. The extraction and assembly of the cores were conducted with assistance from the team at the "Victor Babeș" National Institute, using the semi-automatic TMA Master II system (3D-HISTECH).

The tissue cores were fixed in receptor blocks and stabilized by gently heating the paraffin, then cooled for sectioning. Serial sections of 3–4 μm were made from each TMA block using a microtome. The sections were mounted on adhesive slides (SuperFrost Plus), marked according to the positioning grid, and used for immunohistochemical analysis.

For orientation and quality control, each TMA block included a positive control core and an orientation core (amygdala tissue and an empty core). The blocks were examined for core integrity, proper positioning, and quality of histological sections.

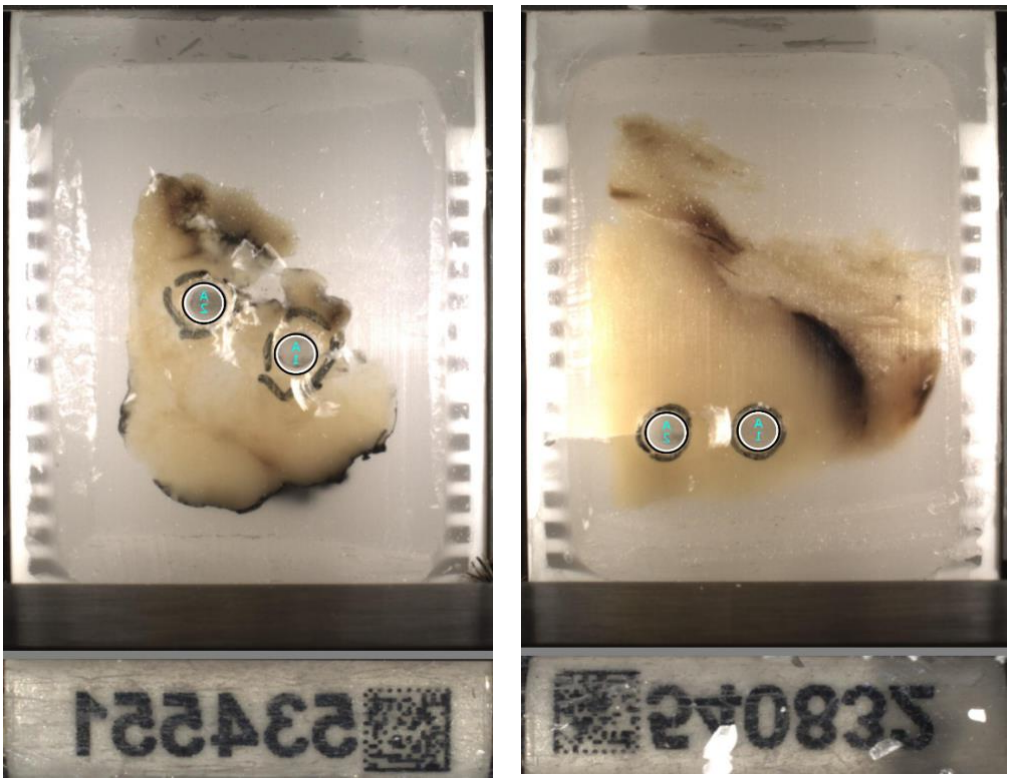

Fig. 3.1 Donor blocks with areas selected for core extraction

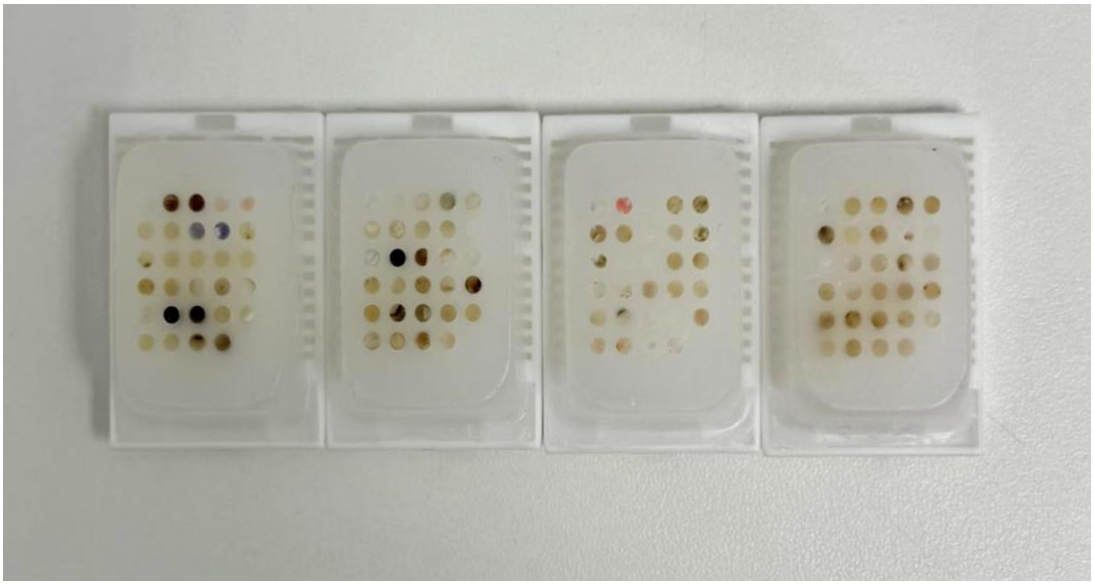

Fig. 3.2 TMA blocks obtained

III.1 Immunohistochemical analysis

For the expression analysis of target proteins, automated immunohistochemistry was performed on the Ventana BenchMark ULTRA platform (Roche Diagnostics), ensuring high standardization and reproducibility. The 3–4 μm sections of the TMA blocks were mounted on SuperFrost Plus slides and incubated at 60°C for 1 hour.

The IHC procedure included automatic dewaxing and rehydration, antigen retrieval (Ultra CC1, 95–100°C), endogenous peroxidase blockade, incubation with primary antibodies at 37°C, and detection with OptiView or ultraView DAB kits, followed by hematoxylin counterstaining. Finally, the slides were washed, dehydrated, and mounted. Positive and negative external controls were used for each antibody. The slides were then morphologically evaluated, scored, and recorded in the database.

III.2 Statistical analysis

Statistical analysis was conducted using IBM SPSS Statistics version 25.0 (IBM Corp., Armonk, NY, USA). The $p<0.05$ values were deemed statistically significant.

The data were categorized into categorical data (nominal or ordinal), represented by frequencies and percentages, and continuous (numeric) data, represented by mean ± standard deviation (SD) for normal distributions or by median and interquartile range (IQR) for non-normal distributions.

To analyze the association between categorical variables, the exact Fisher test was utilized, depending on the expected frequencies.

Kaplan–Meier analyses were employed to evaluate differences in overall survival (OS) between groups based on the factors examined. Overall survival was estimated as mean or median, as appropriate, with accompanying 95% confidence intervals and 3-year survival data. The differences between the groups were statistically tested using the Log-Rank test.

For mortality risk analysis, Cox regression models with proportional hazards were applied in both univariate and multivariate analyses. The impact of each variable was expressed as the hazard ratio (HR), along with the 95% confidence interval and the corresponding p-value. The models were assessed for goodness-of-fit and statistical significance.

IV. Synthesis of scientific research studies (special section)

IV.1 From Biopsy to Diagnosis: Large B-Cell Lymphoma in Practice

The diagnosis of high-grade large B-cell non-Hodgkin lymphoma requires a complex approach, as these neoplasms can mimic other types of solid or hematological tumors. Based on the WHO guidelines (HAEM5), we have created a diagnostic algorithm suitable for current practice, focusing on differentiating between lymphomas, carcinomas, sarcomas, and melanomas in the initial step. The process begins with a morphological evaluation, supplemented by immunohistochemical panels. Key markers such as CD45, CD20, CD3, CD79a, and PAX5 are utilized to determine the cell lineage, particularly in cases of CD20-negative B lymphomas. Special attention is also given to plasmablastic or ALK-positive lymphomas, which may necessitate additional markers such as CD138, ALK-1, and MUM1. Genetic tests (FISH) are important for identifying lymphomas with MYC, BCL2, and BCL6 (double or triple hit) rearrangements, but we recognize that they remain difficult to access within the national context.

Evaluating growth patterns (nodular vs. diffuse), architecture, and cell size helps differentiate between DLBCL, Burkitt lymphoma, B-lymphoma, mantle cell lymphoma, and aggressive forms with intermediate features. The Ki-67 proliferation index is a crucial parameter that reflects the tumor's aggressiveness and aids in distinguishing it from indolent lymphomas.

The classification of aggressive B-cell lymphoma subtypes is supported by the Hans algorithm, which uses the markers CD10, BCL6, and MUM1 to differentiate the germinal center (GCB) phenotype from the activated phenotype (ABC), each of which has distinct therapeutic implications. Additionally, the BCL2 and c-MYC markers allow for the identification of double-expression lymphomas, which are associated with a reserved prognosis.

In conclusion, diagnosing high-grade B lymphomas requires the rigorous integration of morphological, immunophenotypic, and genetic data. Modern algorithms and extensive marker panels significantly enhance diagnostic accuracy and facilitate treatment individualization, thereby improving patients' prognosis.

IV.2 Evaluation of BCL2, c-MYC, and Ki67 expression in aggressive B-cell non-Hodgkin lymphoma

The second research study involved 66 patients diagnosed with diffuse large B-cell lymphoma, having a median age of 61.8 years and a male predominance of 59.1%. The application of the Hans algorithm revealed a predominance of the ABC subtype at 65.2%. The expression of biological markers demonstrated a positivity rate of 57.6% for BCL-2, 65.2% for BCL-6, and 7.6% for C-MYC. Additionally, 65.2% of patients had a Ki-67 proliferation index of ≥75%.

Expression of C-MYC, BCL-2, and BCL-6 markers was observed as double or triple in only 7.6% of cases. The GCB subtype showed a significant association with C-MYC expression, whereas the ABC subtype was prevalent in cases lacking this marker. No significant correlations were found between the expression of C-MYC, BCL-2, or Ki-67 markers and other clinical characteristics, including age, sex, disease stage, or IPI score.

The median overall survival (OS) was 48 months, with a 3-year survival rate of 54.5%. C-MYC expression and IPI score did not significantly impact survival. In contrast, BCL-2 expression indicated a trend toward reduced survival, and a Ki-67 ≥75% significantly correlated with lower survival ($p<0.001$). Patients with elevated Ki-67 had a median survival of 39 months compared to 76 months for those with expression below 75%.

Additionally, age ≥60 years was associated with significantly lower survival rates (41 vs. 68 months, $p=0.010$). The only case with double expression C-MYC/BCL-2 had a survival of 1 month, which, although statistically significant ($p<0.001$), has limited relevance due to the small number of cases.

Predictive analysis of mortality indicated that only age greater than 60 years and high expression of Ki-67 were significant independent predictors ($p<0.05$), associated with increased risks of death of 2.36 and 3.89 times, respectively.

IV.3 PD-L1, PD-1 Expression, and Micromedium in Aggressive B-Cell Non-Hodgkin Lymphoma

The third study was conducted using the same sample of patients, but with the aid of multi-tissue blocks, we examined the immunohistochemical markers PD-L1, PD-1, CD68, CD4, and CD8.

PD-L1 expression was detected in tumor cells in 6 cases (9.1%)—with only one case exceeding 50% and five cases above 1%. Among stromal immune cells, 39 cases (59%) exhibited positive expression. For PD-1, 7.6% of patients demonstrated expression in tumor cells, while 40.9% showed expression in stromal cells. CD68, CD4, and CD8 markers were positive in 23, 34, and 14 cases, respectively.

Statistical analysis did not reveal significant correlations between PD-1 expression and most clinical parameters (age, stage, IPI score), except for gender: women exhibited significantly higher PD-1 expression in immune cells (55.6% vs. 30.8%, $p=0.044$). Regarding PD-L1, expression in tumor cells was significantly associated with morphology ($p=0.015$): anaplastic lymphomas were more frequently positive, while centroblastic lymphomas were negative.

The expression of CD68, CD4, and CD8 markers showed no statistically significant correlations with clinical parameters, except for two notable exceptions. CD68 was significantly associated with the Ann Arbor stage ($p=0.040$), being more frequently expressed in stages I–II. Additionally, CD68 expression was lower in centroblastic lymphomas and higher in high-grade lymphomas ($p=0.010$).

These data suggest a potential prognostic value of PD-1, PD-L1, and CD68 expression in DLBCL morphological subtypes and staging, but this requires validation in studies with large groups.

V. Conclusions and personal contributions

Conclusions

In the current context of pathology, the rapid pace of scientific progress makes the diagnosis of diffuse large B-cell lymphoma primarily reliant on identifying large lymphoid cells and confirming their B-cell origin through immunostaining. Over the last two decades, significant advancements in understanding the genetic characteristics of large cell lymphomas have enabled a more precise classification. Consequently, it is now more crucial than ever for pathologists to uphold modern standards of diagnostic accuracy when evaluating these cases.

The diagnosis and classification of large B-cell lymphomas remains a complex yet essential process in modern hematopathology. The 2022 WHO classification introduced nuanced revisions that enhance diagnostic accuracy, guide therapeutic decisions, and reflect advances in our understanding of the genetic and phenotypic heterogeneity of these neoplasms. Although DLBCL is the most common subtype, it often presents diagnostic challenges due to morphological overlaps with other aggressive lymphomas and non-hematopoietic malignancies.

The precise subclassification of DLBCL relies on a multidisciplinary approach that integrates morphology, immunohistochemistry, cytogenetics, and molecular diagnostics. Key distinctions among GCB, ABC, and high-grade B lymphomas—including double- or triple-rearrangement variants (double-hit and triple-hit)—are crucial for determining prognosis and personalizing treatment. The use of diagnostic algorithms, such as the Hans classifier, along with the careful application of complementary techniques like FISH and the evaluation of the Ki-67 proliferation index, serves as indispensable tools in the field of histopathological diagnosis.

As the diagnosis of lymphomas evolves, it is essential to maintain a high standard of accuracy and to update knowledge in line with the new classification criteria to enhance patient clinical outcomes.

Immunohistochemical analysis of markers C-MYC, BCL2, and Ki-67 within the studied cohort highlighted the value of these parameters as useful tools in the prognostic assessment of patients with DLBCL. However, the absence of advanced genetic and molecular tests, such as fluorescent in situ hybridization (FISH) or genomic sequencing,

represented a significant limitation of the study, restricting the possibility of a complete molecular characterization of the analyzed cases. In this context, immunohistochemistry is emerging as a practical and accessible solution with clinical applicability for identifying relevant predictive factors, especially in centers where the infrastructure for molecular tests is limited or non-existent.

The GCB subtype demonstrated a significant association with positive C-MYC expression, and a Ki-67 proliferation index ≥75% correlated with markedly reduced overall survival. These findings reinforce the significance of these biomarkers in risk stratification and in supporting personalized therapeutic decisions.

Although the expression BCL2 did not reach the threshold of statistical significance in correlation with overall survival at three years, the observed trend suggests a potential prognostic role, especially when assessed alongside other markers. This hypothesis needs validation through further studies in large samples.

The study regarding the expression of PD-L1 and PD-1 in non-Hodgkin's large B-cell lymphoma demonstrated a significant association between tumor expression of PD-L1 and the morphological type of DLBCL, with anaplastic lymphomas correlating with increased expression of PD-L1. This finding supports the hypothesis of a potentially favorable response to anti-PD-1/PD-L1 immunotherapy in this morphological subtype, emphasizing the value of PD-L1 as a possible predictive biomarker.

PD-1 expression in stromal immune cells was more prevalent in female patients, indicating potential sex-related immunological differences in DLBCL that could affect the immune response and warrant further investigation.

CD68-labeled macrophage infiltration is associated with high-grade morphological subtypes and advanced Ann Arbor stages, suggesting a potential role for tumor-associated macrophages in disease progression and tumor immunosuppression. This aspect supports the integration of markers from the tumor microenvironment into prognostic evaluations and future therapeutic strategies.

Although the expression of CD4 and CD8 markers did not show statistically significant correlations with clinical parameters, the overall T infiltration profile highlights an immune imbalance with potential functional impact, dominated by CD8-positive T cells. The expression of immune checkpoints, including PD-1, on these cells suggests an exhausted phenotype, which could limit the effectiveness of the antitumor immune response.

Personal contributions (originality of the thesis)

One of the main contributions of this thesis is the development of a practical diagnostic algorithm for diffuse high-grade B-cell lymphoma, based on the update from the 5th edition of the WHO classification of hematopoietic tumors. This algorithm integrates morphological, immunohistochemical, and clinical criteria to effectively guide anatomical pathologists and clinicians in establishing an accurate, reproducible, and prognostic-oriented diagnosis, thus contributing to the standardization of the evaluation of these cases in current medical practice.

In the study, we implemented the TMA technique by selecting and processing 66 cases of DLBCL to perform a uniform and comparable immunohistochemical analysis. This approach allows the simultaneous evaluation of several markers under the same technical conditions and reduces pre-analytical variability. By using TMA, we have provided a solid basis for further statistical and biological correlations and demonstrated the feasibility of the method in resource-constrained laboratories.

An important contribution of this work was to highlight significant correlations between the expression of BCL2, c-MYC, and Ki-67 markers and the morphological aspects, specifically the prognosis of patients. We have shown that the concomitant expression of BCL2 and c-MYC defines cases with a "double expressor" phenotype, which is associated with a negative prognosis. Additionally, an increased Ki-67 proliferative index has been linked to aggressive forms of the disease, supporting the use of these markers in risk assessment stratification.

We evaluated for the first time in a national cohort the expression of PD-1 and PD-L1 markers in both tumor and stromal cells within DLBCL lymphoma. The analysis revealed a heterogeneous expression pattern, with significant differences observed between histological subtypes, contributing to our understanding of the complexity of the immune microenvironment. The results have implications for selecting patients eligible for immunotherapy and suggest an active role of the PD-1/PD-L1 pathway in the pathogenesis of certain DLBCL subgroups.

Another important personal contribution involved the quantitative and qualitative characterization of immune cells from the tumor microenvironment, including helper T lymphocytes (CD4), cytotoxic lymphocytes (CD8), and macrophages (CD68). We conducted a comparative evaluation between cases with typical morphology and those with anaplastic features, demonstrating the influence of immune infiltrate on clinical outcomes.

This data enhances our understanding of tumor-microenvironment interactions and can guide future directions in immuno-oncology research.

A distinctive element of this thesis is the application and adaptation of the Hans algorithm for the molecular classification of DLBCL into GCB and ABC subtypes, using exclusively the immunohistochemical markers CD10, BCL6, and MUM1. We adjusted the positivity thresholds based on the literature and demonstrated the algorithm's usefulness in the absence of genetic testing (FISH), thereby providing an accessible and efficient method within the context of Romanian medical practice.

Multivariate statistical analysis (Cox model) enabled the identification of independent prognostic factors, with age ≥60 years and a Ki-67 index ≥75% proving to be significant predictors for overall survival. This finding supports the inclusion of these parameters in the standard assessment of DLBCL cases and has the potential to guide treatment decisions, particularly in the absence of advanced genetic testing.

We developed a comprehensive database that integrates clinical (age, sex, location), morphological, immunohistochemical, and survival parameters for each of the 66 cases. This database has been utilized for descriptive and inferential statistical analyses, serving as a valuable tool for validating the formulated hypotheses and for future applications in clinicopathological research.

In the absence of available FISH tests in many medical centers, we have demonstrated the usefulness of immunohistochemical expression of BCL2 and c-MYC markers as a screening method for identifying cases with double-hit potential. This strategy allows patients to be referred for further genetic investigations only when the expression is concomitant, helping to optimize costs and prioritize high-risk cases.

We highlighted a significantly increased expression of PD-L1 in morphologically anaplastic subtypes of DLBCL, suggesting a possible mechanism of immune evasion in these aggressive forms. This observation may provide a foundation for introducing anti-PD-L1 therapies in the personalized treatment of these patients and complements the limited data in the literature regarding the correlation between morphology and immune expression.

This work represents not only an academic approach but also a response to the need to reduce the uncertainty surrounding a harsh diagnosis and to contribute, even modestly, to improving the diagnostic and therapeutic path for patients. Thus, the doctoral thesis entitled "Morphological and immunophenotypic variability of high-grade B-cell lymphomas" aims to bring even more clarity to the "gray" areas of diagnosis, support the medical team with

useful information, and, above all, assist the patient in the fight against a disease that does not always allow time.

Selective bibliography

1. Eman Mohamad Ibrahim1, S.R., Shaimaa El-Ashwah2, Maryan Waheeb Fahmi3* and Afaf Taha Ibrahiem1, *Programmed death ligand 1 expression in diffuse large B cell lymphoma: correlation with clinicopathological prognostic factors.* Journal of the Egyptian National Cancer Institute, 2023.

2. Buruiana, S., *Incursion into Non-Hodgkin's Lymphomas Associated with Antiphospholipid Syndrome*.

3. Craig, A., et al., *Utility of PD-1, PD-L1, and IDO-1 Stains in Ocular Extranodal Marginal Zone Lymphoma (MZL) and Diffuse Large B-cell Lymphoma (DLBCL).* Applied Immunohistochemistry & Molecular Morphology, 2024. **32**(8): p. 395-399.

4. Romania, M.S. *National-Plan-to-Control-Cancer*. 2022; Available from: https://www.ms.ro/media/documents/Plan-National-de-Combatere-a-Cancerului.pdf.

5. Patrascu, A.M., et al., *The prognostic role of Bcl-2, Ki67, c-MYC and p53 in diffuse large B-cell lymphoma.* Rom J Morphol Embryol, 2017. **58**(3): p. 837-843.

6. Dong, L., et al., *Is first-line treatment with polatuzumab vedotin-rituximab-cyclophosphamide, doxorubicin and prednisone (pola-R-CHP) for previously untreated diffuse large B-cell lymphoma cost-effective in China? A cost-effectiveness analysis using a Markov model.* BMJ Open, 2025. **15**(1): p. E086251.

7. Linschoten, M., et al., *Rationale and design of the HOVON 170 DLBCL-ANTICIPATE trial: preventing anthracycline-induced cardiac dysfunction with dexrazoxane.* Cardiooncology, 2025. **11**(1): p. 8.

8. Yimpak, P., et al., *Immunohistochemistry-based investigation of MYC, BCL2, and Ki-67 protein expression and their clinical impact in diffuse large B-cell lymphoma in upper Northern Thailand.* PLoS One, 2024. **19**(7): p. E0307253.

9. Talaulikar, D., et al., *DETAILED IMMUNOPHENOTYPING OF B-CELL SUBSETS IN DIFFUSE LARGE B-CELL LYMPHOMA.* Cytometry Part B-Clinical Cytometry, 2013. **84**(6): p. 423-423.

10. Zhang, S.X., *PD-1 Expression Is Upregulated in Diffuse Large B-Cell Lymphoma Transformed from Marginal Zone Lymphoma and Significantly Higher in Comparison with de novo Diffuse Large B-Cell Lymphoma.* Modern Pathology, 2019. **32**.

11. Alig, S.K., et al., *Evolving molecular classification of aggressive B-cell lymphoma.* Histopathology, 2025. **86**(1): p. 94-105.

12. Shao, Y., et al., *Artificial Intelligence-Driven Precision Medicine: Multi-Omics and Spatial Multi-Omics Approaches in Diffuse Large B-Cell Lymphoma (DLBCL).* Biosci Front (Landmark Ed), 2024. **29**(12): p. 404.

13. Tanabe, A., J. Ndzinu, and H. Sahara, *Development and Validation of a Novel Four Gene-Pairs Signature for Predicting Prognosis in DLBCL Patients.* Int J Mol Sci, 2024. **25**(23).

14. Chapuy, B., et al., *DLBclass: A Probabilistic Molecular Classifier to Guide Clinical Investigation and Practice in DLBCL.* Blood, 2024.

15. Vareli, A., et al., *Systems biology-enabled targeting of NF-kappaB and BCL2 overcomes microenvironment-mediated BH3-mimetic resistance in DLBCL.* bioRxiv, 2024.

16. Zanelli, M., et al., *A Diagnostic Approach in Large B-Cell Lymphomas According to the Fifth World Health Organization and International Consensus Classifications and a Practical Algorithm in Routine Practice.* International Journal of Molecular Sciences, 2024. **25**(23).

17. Shold, J., et al., *EBV Positive Diffuse Large B Cell Lymphoma with Negative Pan-B Cell Markers, Case Report, and Literature Review.* Case Reports in Hematology, 2024. **2024**.

18. Collinge, B., et al., *The impact of MYC and BCL2 structural variants in tumors of DLBCL morphology and mechanisms of false-negative MYC IHC.* Blood, 2021. **137**(16): p. 2196-2208.

19. Jiang, Y.Y., et al., *Primary mediastinal large B cell lymphoma with coexisting aberrations of C-MYC and BCL-2: a case report and literature review.* Medical Molecular Morphology, 2020. **53**(2): p. 124-129.

20. Mehta, A., et al., *Double Hit and Double Expresser Diffuse Large B Cell Lymphoma Subtypes: Discrete Subtypes and Major Predictors of Overall Survival.* Indian J Hematol Blood Transfuse, 2020. **36**(4): p. 627-634.

21. Mohammed, A.A., et al., *C-MYC and BCL2: Correlation between Protein Over-Expression and Gene Translocation and Impact on Outcome in Diffuse Large B Cell Lymphoma.* Asian Pac J Cancer Prev, 2019. **20**(5): p. 1463-1470.

22. Maillie, L., et al., *Prolonged neurologic symptoms following immune effector cell-associated neurotoxicity syndrome in patients with large B cell lymphoma treated with chimeric antigen receptor-modified T cell therapy.* Transplant Cell Ther, 2025.

23. Hartmann, S. and M.L. Hansmann, *Large B-Cell Lymphoma Rich in PD-1+ T Cells An Overlooked Subtype of Diffuse Large B-Cell Lymphoma?* American Journal of Clinical Pathology, 2014. **142**(2): p. 142-143.

24. Marinaccio, C., et al., *Microvascular Density, CD68 and Tryptase Expression in Human Diffuse Large B-Cell Lymphoma.* Leukemia Research, 2014. **38**(11): p. 1374-1377.

List of published scientific papers

1. **Halcu, G.**; Evsei-Seceleanu, A.; Cerbu, M.; Bara, M.A.; Turbatu, A.; Ceausu, M.C. *From Biopsy to Diagnosis: Navigating Aggressive B-Cell Lymphomas in Practice.* Medicine 2025, 61, 842. ISI indexed journal with FI 2.4/2023, Q1, chapter 7, p. 46−60
   https://doi.org/10.3390/medicina61050842
   https://www.mdpi.com/1648-9144/61/5/842

2. **Halcu, G**.; Evsei-Seceleanu, A.; Tapoi, D. A.; Cerbu, M.; Barta, C.; Ceausu, M. *Correlations Between Immunophenotypic Markers and Clinical Progression in Romanian Patients Diagnosed with Diffuse Large B-cell Lymphoma*. Medicine 2025, 61, 948. ISI indexed journal with FI 2.4/2023, Q1, chapter 8, p. 61−75
   https://doi.org/10.3390/medicina61060948
   https://www.mdpi.com/1648-9144/61/6/948

3. **Halcu, G. et al**; *PD-L1 expression and tumor microenvironment dynamics in diffuse large B-cell lymphoma: immunophenotypic insights*. Journal of Medicine and Life 2025. Pubmed, chapter 9, p. 79−92
   https://doi.org/10.25122/jml-2025-0078
   https://medandlife.org/issues/

4. Dumitru, A.V.; Țăpoi, D.A.; **Halcu, G**.; Munteanu, O.; Dumitrascu, D.-I.; Ceaușu, M.C.; Gheorghișan-Gălățeanu, A.-A. *The Polyvalent Role of CD30 for Cancer Diagnosis and Treatment.* Cells 2023, 12, 1783. ISI indexed journal with FI 5.1/2023, Q1, chapter 3, p. 30−31
   https://doi.org/10.3390/cells12131783
   https://www.mdpi.com/2073-4409/12/13/1783

Special thanks

I want to extend special thanks to Associate Prof. Dr. Ceaușu Mihail, the coordinator of my doctoral thesis, for the opportunity offered and the constant support received throughout the entire research period.

Sincere thanks to our fellow technicians from Colțea Clinical Hospital for their professionalism and availability in carrying out practical activities. At the same time, I would like to express my gratitude to my fellow doctors from the Pathological Anatomy Service of Colțea Hospital for their support and understanding.

In particular, I would like to thank my family and loved ones, who have supported me unconditionally and provided me with the necessary balance to complete this academic journey.

„We don't really know a thing until we have researched it in depth."

Descartes