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DOCTORAL THESIS

Correlations of clinical-morphological and genetic studies in the evaluation of prognostic and diagnosis of non-Hodgkin malignant large B-cell lymphomas

Thesis abstract

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TITLE OF CONTENTS

Keywords: Lymphoma, Non-Hodgkin, large B-cell, diffuse, onco-protein MYD88, onco-protein PIM1, machine learning, Ki67 antigen, CD5 antigen, prognosis.

The PhD thesis includes:

- 282 pages, of which 59 are the General Part;
- 95 figures, of which 2 are in the General Part;
- 102 tables, of which 5 are in the General Part;
- 503 bibliographic references.

Note: in this sumary, the content has been kept in the same form as in the doctoral thesis.

INTRODUCTION

The present study aims to describe the lymphoid tumoral pathology (large B-cell non-Hodgkin's lymphomas) following their clinical, histopathological, immunohistochemical and genetic characteristics. For a good understanding of the mechanisms of development and evolution of these entities, these data are imperatively necessary, both for the establishment of clear criteria for histopathological diagnosis and for the identification of prognostic factors with a role in patient survival.

By consulting the current specialized literature, we noticed a special concern of several researchers on the early diagnostic criteria of large B-cell lymphomas, as well as the importance of prognosis in their evolutionary stages. Thus, the chosen theme represents a current topic, in which, until now, not much data is known.

Exome sequencing studies have recently illustrated that despite the use of different sequencing approaches and clustering algorithms – the genetic landscape of large B-cell lymphomas can be used to subclassify them with broad concordance, influencing the prognosis and therapeutic conduct of the disease. The motivation for this study is primarily due to the high mortality in large B-cell non-Hodgkin's lymphomas that requires the use of accurate diagnostic methods with high reliability.

Therefore, it is necessary to identify some biomarkers to predict the evolution and prognosis, which would allow a personalized therapy.

The validation of a standardized panel of genetic mutations to classify patients into risk groups according to prognosis and subsequently to allow the choice of targeted therapy represents an element of originality in the management of large B-cell lymphomas.

CURRENT STATE OF KNOWLEDGE

1. Anatomy and histophisiology of lymphoid organs

Lymphoid tissues are the sites where precursor cells mature into immunocompetent lymphoid cells and where immune reactions to antigens occur. Lymphoid tissues and the stages of differentiation and maturation of lymphocytes have an anatomy - they occur in certain places in the body. They have an architecture—each lymphoid tissue is organized in a specific way, and cell differentiation and reactions occur at specific sites within this organized tissue. They have a specific cell morphology—cells change size, shape, and other characteristics as they mature and react to antigen and other stimuli. They undergo specific genetic and biological changes—lymphoid cells change their genes, gene expression, and the proteins they produce and respond to at different stages of differentiation and maturation. Understanding these normal structures and their changes during lymphoid cell development and activation and during immune responses is important for pathologists who must diagnose reactive and neoplastic states of lymphoid tissues and cells.

Superimposed on this anatomy of lymphoid tissue is the biology of the immune system. The function of the immune system is to defend against infection. Its cellular components include

phagocytic cells (neutrophils, monocytes, and histiocytes or macrophages), lymphocytes (T cells, B cells, and natural killer [NK] cells), and antigen-presenting cells (histiocytes, dendritic cells, and B cells). There are two distinct types of immune reactions: innate or natural immune responses and acquired or adaptive immune responses.

Innate immune responses are carried out by phagocytes, dendritic cells, NK cells and some T cells, including gamma-delta T. cells, which respond in the same way regardless of previous exposure to the antigen.

Adaptive immune responses involve antigen-specific T cells and B cells and are modified by prior antigen exposure. Antigen recognition in the innate immune system is mediated by receptors encoded in germline DNA. Since the existence of the first multicellular organisms, these receptors have evolved to recognize a limited number of highly conserved structures that are present on common pathogens—the so-called pathogen-associated molecular patterns—but are not present on host cells. These include bacterial lipopolysaccharides, yeast cell wall mannans, bacterial DNA, and others. In contrast, antigen recognition in the adaptive immune system is mediated by somatically generated receptors in B and T cells, producing a wide variety of surface receptors, only some of which have useful specificity. Those that are dangerous (ie, having anti-self specificity) must be selected against those that are useful (ie, specific for pathogens) must be selected by clonal expansion upon antigen exposure. The adaptive immune response improves in efficiency and specificity during the individual's lifetime due to repeated exposure to the antigen, but by definition this cannot be passed on to offspring.

Another major difference between innate and adaptive responses is that innate immune cells perform their effector functions immediately after receptor engagement, whereas cells of the adaptive response first proliferate in response to antigen. In addition to the rapid recognition and control of pathogens, cells of the innate immune system initiate and regulate adaptive immune responses through antigen presentation and activation of T cell and B cell signals.

1.1. Normal lymphoid tissues

Lymphoid tissues are divided into two major compartments, depending on the stages of lymphoid cell differentiation and functional interactions: central or primary lymphoid tissues and peripheral or secondary lymphoid tissues. The central lymphoid tissues are the bone marrow and the thymus. These organs contain precursor lymphoid cells and support the initial process of antigen-independent differentiation from immature cells to the mature stage, where they can perform their function in response to antigens. The peripheral or secondary lymphoid organs are the lymph nodes, spleen, and mucosa-associated lymphoid tissue (MALT), where mature lymphoid cells encounter antigens and develop various types of immune responses. These compartments are highly organized microenvironments of different cell populations, vascular structures, and stromal components that maximize selective interactions between lymphocytes and antigens to initiate and extend immune responses.

2. General considerations, classification, staging and histopatology of large B-cell non-Hodgkin's lymphoma

2.3. WHO classification and staging of large B-cell non-Hodgkin lymphomas B

The new WHO edition recognizes 17 specific entities as large B-cell lymphomas other than DLBCL, NOS grouped according to their genetic characteristics, specific clinical setting, or particular particular site of origin (extranodal):

- Large B-cell lymphoma with IRF4 rearrangement
- Diffuse large B-cell lymphoma, NOS
- T-cell/histiocyte-rich large B-cell lymphoma
- Primary large B-cell lymphoma of immune-privileged sites; Primary diffuse large B-cell lymphoma of the central nervous system.
- Diffuse large B-cell lymphoma/High-grade B-cell lymphoma with MYC and BCL2 rearrangements
- High-grade B-cell lymphoma with 11q aberration
- Lymphomatoid granulomatosis
- Primary cutaneous diffuse large B-cell lymphoma, leg-type
- Diffuse large B-cell lymphoma, EBV-positive
- Diffuse large B-cell lymphoma associated with chronic inflammation/fibrin
- Mediastinal (thymic) large B-cell lymphoma
- Plasmablastic lymphoma
- Fluid-overload large B-cell lymphoma
- Intravascular large B-cell lymphoma
- ALK-positive large B-cell lymphoma
- High-grade B-cell lymphoma, NOS
- Mediastinal grey zone lymphoma

In most of these categories, biological concepts and diagnostic strategies have remained largely unchanged; however, some of the names have been changed since the 4th (revised) edition, such as the name diffuse large B-cell lymphoma to large B-cell lymphoma, recognizing that a diffuse growth pattern does not necessarily part of the disease definition or that a growth pattern cannot be assessed in some entities.

Lymphoma types/entities are defined by morphologic, immunophenotypic, and clinical criteria, but none of these are absolutely specific and defining. Therefore, a hierarchical line of decision-making is inevitable. The morphological and/or clinical characteristics that define the entity/type, respectively, override the genetic characteristics in such cases. Similarly, a feature of immune deficiency/dysregulation overrides other defining parameters (eg, CNS location of DLBCL associated with immune deficiency/dysregulation, EBV-positive, HIV-positive).

2.3.4. Histopathological aspects of non-Hodgkin large B-cell lymphomas

Diffuse large B-cell lymphoma (DLBCL), NOS

Diffuse large B-cell lymphoma, not otherwise specified (DLBCL, NOS), is a lymphoma consisting of medium-sized to large B cells with a diffuse growth pattern. This is a morphologically and molecularly heterogeneous entity that does not meet the diagnostic criteria of specific large B-cell lymphoma neoplasms.

2.3.4.2. T-cell/histiocyte-rich large B-cell lymphoma (THRLBCL)

T-cell/histiocyte-rich large B-cell lymphoma (THRLBCL) is an aggressive B-cell lymphoma with <10% large neoplastic B cells, scattered in a diffuse background rich in T cells and histiocytes, and with virtual absence of small B cells. A subset of cases shows marked clinical, immunophenotypic, and molecular overlap with nodular lymphocyte predominant Hodgkin lymphoma (NLPHL).

2.3.4.3. Primary large B-cell lymphoma of immune-privileged sites; Primary diffuse large B-cell lymphoma of the central nervous system.

Primary large cell B-cell lymphomas (LBCL) of immune-privileged sites (IP-LBCL) comprise large B-cell lymphomas that arise as primary tumours in the central nervous system (CNS), vitreoretina, and testis of immunocompetent patients. Excluded from this category are the lymphomas that arise in the dura and the choroid, lymphomas secondarily involving these sites, and those occurring in immune deficiency/dysregulation-related settings.

2.3.4.4. Primary cutaneous diffuse large B-cell lymphoma, leg-type

Limfomul primar cutanat difuz cu celule B mari, de tip gambier (PCLBCL-LT) este un limfom compus exclusiv din centroblaste și imunoblaste, care apar cel mai frecvent la nivelul piciorului.

2.3.4.5. High grade B-cell lymphoma, NOS

High grade B-cell lymphoma, NOS (HGBL, NOS) represents a heterogeneous category of aggressive mature B-cell lymphomas composed of medium-sized or blastoid cells that do not fit into other defined categories of lymphomas.

PERSONAL CONTRIBUTION

3. The motivation, aim and objectives of the study

In the accurate diagnosis of lymphoid neoplasms, a multidisciplinary approach covering clinical, microscopic and molecular aspects is essential. The choice of this topic derives from the complexity of the clinical manifestations and the frequent overlaps between the histopathological features of the different types of B-cell lymphoma, as well as due to the high mortality in the case of this tumor spectrum that requires the use of accurate diagnostic methods with high reliability. Also, pathogenic data are still insufficient, even despite the many studies carried out to date. The present study aims to identify 4 oncogenic mutations in patients with large B-cell non-Hodgkin lymphomas and validate them in the standard investigation panel with the aim of stratifying patients by risk groups, as well as highlighting possible correlations with clinico-

morphological and genetic variables in order to select the most faithful indicators of prediction and evolution of the disease. The aim is also to identify the fastest and most reliable diagnostic methods, as well as to evaluate the prognostic utility of some biomarkers in these neoplasms.

4. Material and methods

A retrospective study was carried out including a group of 50 patients diagnosed with malignant non-Hodgkin large B cell lymphoma, within the Pathological Clinical Anatomy Service of Constanța and within the Pathological Anatomy Service of Săcele, Brașov, under oncological treatment for this condition. The clinical and paraclinical characteristics of the patients were collected retrospectively from the observation sheets of the patients from the Department of Internal Medicine and Hematology and the registry of the Pathological Clinical Anatomy Service: age at onset, sex, paraclinical data (LDH, viral serology), treatment followed.

Research methods used to carry out the study:

1. The selection of cases from the slides storage of the Constanța's Pathological Clinical Anatomy Service and from the Săcele's Pathological Anatomy Service and the anatomopathological and immunohistochemical re-evaluation - represents the classification of the lesions previously diagnosed as non-Hodgkin's large B-cell lymphoma according to the current published staging and classifications in WHO 2022. All patients were staged using the ECOG Performance Status, the Ann Arbor Staging System and the International Prognostic Index.
2. Immunohistochemical analysis was performed using the biomarkers MYD88 (clone RM306) and PIM1 (clone ST0513), from Novus Biologicals, using the DAB method and following the manufacturer's recommendations, and was performed within the Research Center for the Development of Morphological and Genetic Studies in Malignant Pathology (CEDMOG), "Ovidius" University from Constanța.
3. Biomarker evaluation was performed using both qualitative and quantitative. An intensity-based scoring system was used to quantify the immunohistochemical expression of MYD88.
4. Slide scanning was obtained using the Huron LE120TM 4000XT slide scanner within the Center for Research and Development of Morphological and Genetic Studies in Malignant Pathology (CEDMOG), "Ovidius" University of Constanța.
5. Digital quantification of Ki67 proliferation index was performed using QuPath software (version 0.4.3.).
6. Genomic DNA extraction was performed from paraffin blocks by the method of macroscopic dissection of representative neoplastic tissue and processed using the QiaAmp DNA FFPE Tissue Kit extraction kit (Qiagen, Germany) according to the manufacturer's recommendations. The extracted DNA samples were quantified spectrophotometrically with Nanodrop OneC (ThermoFisher Scientific), determining the concentration and purity by calculating the ratios A260/A280nm and A260/A230nm, but also fluorometrically using Qubit III (Life Technologies) for a more accurate concentration determination.
7. MYD88 and PIM-1 genotyping was performed using the TaqMan SNP Genotyping Assay kit, (AppliedBiosystems), using TaqMan probes specific for the mutations of the 2 genes.
8. Uni- and multivariate statistical analysis of stratified cases, as well as logistic regression, was performed using SPSS software version 26.0.

5. Study 1 – Comparative study between the classical conventional method and the evaluation by means of artificial intelligence of the Ki67 proliferation index in large B-cell non-Hodgkin's lymphomas with gastrointestinal localization

5.1. Introduction

The gastrointestinal tract is the most common site of extranodal non-Hodgkin lymphoma, accounting for 20% to 40% of all extranodal lymphomas. The majority of non-Hodgkin's lymphomas involving the gastrointestinal tract belong to the B-cell proliferative lineage, of which diffuse large B-cell lymphoma is the most common subtype, regardless of location. With the recent development of digital whole slide imaging (WSI), it is now possible to automatically identify the histopathological features of lymphomas. One method of digital pathology is the process of using slide scanners to convert glass slides of histopathological specimens into high-resolution digital images, which are then interpreted and used to generate pathological data. QuPath is software that provides researchers with powerful, continuous batch processing functionality and an extensible platform with which to develop and share new algorithms to analyze complex tissue images. However, studies on the use of digital pathology to identify lymphoma are still limited to only determining whether a tumor proliferation is present or absent.

The main aim of this current study is to investigate the diagnostic utility of PD using QuPath software (version 0.4.3) in evaluating the prognostic significance of Ki67 in patients with gastrointestinal large B-cell lymphomas, to compare the values obtained between the conventional method of assessment and evaluation with the help of artificial intelligence (AI) and to observe the prognostic role in patient survival of the parameters studied in a group of patients diagnosed within two centers from Romania, observing their associations and correlations.

5.4. Results

The mean age of the patients was 56.73 years (SE 3.833) with extremes between 32 and 86 years. The male gender was the most affected, observed in 60% of cases. A statistically significant association was observed between the presence of secondary anemia and young patients, as opposed to older ones ($p = 0.035$). The mean age of patients with DLBCL, NOS, was higher (58.15 years) compared to the mean age of those with HGBL, NOS (47.50 years), without a statistically significant difference ($p = 0.305$).

The most frequent localization of large B-cell non-Hodgkin's lymphomas in the gastrointestinal tract was in the stomach (33.33%) and in the small intestine (33.33%). Also, the most frequent localizations of large B-cell non-Hodgkin's lymphomas in the gastrointestinal tract in both men and women, with the same percentage, were in the stomach (33.33%) and small intestine (33.33%).

The most common comorbidities encountered were secondary anemia (93.33%) and hyperuricemia (20%). As a significant risk factor for the development of the disease, only one case presented Helicobacter pylori infection and DLBCL, NOS, located in the stomach (6.67%).

In cases where the cell of origin could be identified (86.67%), a predominance of the germinal center B cell-like subtype (GCB) was observed (84.62%).

The mean value of serum LDH was 731.07, with limits between 164 and 3956. In 66.67% of cases a value above the normal limit was observed. A statistically significant difference was observed, in that older patient age correlated with increased LDH values (p = 0.040).
At the end of the study, 10 patients died (66.67%). The median survival of patients in the group who died was 32.07 weeks (1-126.09 weeks).
Most patients (73.3%) had a low IPI score (0-2), and more than half of patients (53.3%) had a high ECOG prognostic score (2-4). Ann Arbor staging highlighted all cases in the high-grade category (III–IV). Almost all patients with lymphoma located in the small intestine had a high IPI score (3–5) compared to the other locations, and in contrast, all patients with lymphoma located in the stomach had a low IPI score (0–2), with statistically significant significance (p = 0.012).
The distribution at the level of the digestive tract showed an increased frequency of them at the level of the stomach and small intestine (33.33% each), less evident at the proximal level (oropharyngeal 13.33%). Depending on the lesional topography, an increased survival was observed in the locations in the large intestine (94.20 weeks) as opposed to the other locations (p = 0.011). However, location is not an independent risk factor predicting mortality (p = 0.054).
The mean proliferation index (Ki67) was 58.33% with values ranging from 10% to 85%. However, assessment by conventional method (visual/optical microscopy) gives lower Ki67 values than automated digital image analysis. After stratifying the cases, an increased proliferation index was observed in the majority of cases (53.33%). This aspect was associated with the older age of the patients (p = 0.045). The coefficient of agreement between the conventional method and the AI method indicates an excellent level of reliability (ICC1–0.970, ICC2–0.990). Multivariate analysis revealed that in cases where the Ki67 proliferation index is high (>70%), the IPI score is an important risk factor predicting mortality (HR = 10.597, p = 0.033).

5.5. Discussions

In our study, DLBCL, NOS, was the most common subtype in the studied patient group (86.67%), consistent with the results obtained and data provided by WHO 5th Edition, 2022. According to previous studies, the most common location for gastrointestinal NHL is the stomach (60-75% of all cases), followed by the small intestine and ileocecal region, and two of the most common diagnoses are diffuse large B-cell lymphoma (DLBCL) and zone lymphoma marginal (MALT). Our research supports this: the most common localization of patients diagnosed with DLBCL, NOS, in the gastrointestinal tract was in the stomach (33.33%) and small intestine (33.33%).

In terms of molecular subtypes, the GCB (germinal center B cell) subtype has a frequency of approximately 60%, while the ABC (activated B cell) subtype has a frequency of approximately 25–30%. Our research identified cases where the cell of origin could be determined (86.67%), with a greater predominance of the GCB subtype (84.62%).

Recent studies have shown that in lymphoma, serum LDH correlates strongly with higher levels of cell-free tumor DNA and could be a surrogate of increased circulating tumor cells. The present study demonstrates that in 66.67% of cases a value above the normal limit (>250) was observed and a statistically significant difference, consisting in the fact that the advanced age of the patients correlated with the increased values of serum LDH (p = 0.040) . In our research, in

terms of patient survival according to this parameter, no major differences were observed – 31.39 weeks for increased values and 33.66 for normal values.

According to a study by Prochazka et al., in univariable time-to-event analysis, elevated uric acid levels were associated with worse PFS (progression-free survival; relative risk (HR)) and survival (OS)) worse. In our study, one of the most common comorbidities encountered was hyperuricemia (20%), but it did not correlate with a worse survival rate. The nuclear protein Ki-67 is involved in the control of cell proliferation, and its expression has been commonly used as a marker to assess the proliferative activity of lymphoma. Its predictive significance for lymphoma is still undefined and insufficient, however. In our study, after stratifying the cases, an increased proliferation index was observed in most cases (53.33%), and this aspect was associated with the advanced age of the patients ($p = 0.045$). Median survival was similar (31.04 weeks and 33.62 weeks, respectively) regardless of whether the Ki67 proliferation index was classified as high or low. According to a study by Broyde et al., in diffuse large B-cell lymphoma, a cut-off value of 70% can distinguish patients with good and poor prognosis when combined with other prognostic factors such as low IPI score and disease type " bulky". Similarly, our study showed that in cases of large B-cell lymphoma in which the Ki67 proliferation index is high (>70%), the IPI score is an important risk factor in predicting mortality. However, the intra- and interobserver variability of Ki-67, which is dependent on the heterogeneity of the tumor and the area under examination, discourages its validation as a prognostic factor. In our study, the coefficient of agreement between the conventional method and the AI method indicates an excellent level of reliability (ICC1–0.970, ICC2–0.990).

5.6. Conclusions

In conclusion, our study showed that in cases of large B-cell lymphoma in which the Ki67 proliferation index is high (>70%), the IPI score is an important risk factor predicting mortality, without a statistically significant correlation between Ki67 expression analyzed with conventional method or AI method and ECOG performance status or Ann Arbor staging.

Despite the challenging diagnostic histopathological screen for practicing pathologists, as different forms of lymphomas show modest heterogeneity in their histological findings, automated identification has been shown to be more objective and reproducible. Evaluation of Ki67 in large B-cell non-Hodgkin's lymphomas using QuPath software can achieve the goal of increasing the productivity of pathologists and demonstrate the feasibility of integrating an automated lymphoma diagnostic screen to aid in their more accurate diagnosis, in future pathologic anatomy workflow.

6. Study 2 – Molecular profiles and immunohistochemical expressions of MYD88 and PIM1 genes in large B-cell non-Hodgkin lymphomas

6.1. Introduction

The morphological and/or clinical frameworks that define the entity/type override the genetic characteristics in such cases. Similarly, an immune deficiency/dysregulation setting overrides other defining parameters (eg, CNS location of DLBCL associated with immune deficiency/dysregulation, EBV-positive, HIV-positive setting). These are enriched for BCR pathway mutations such as in MYD88, CD79B, PIM1 and PRDM1 encoding BLIMP1. Primary myeloid differentiation response protein 88 (MYD88) is an important adapter molecule in toll-like receptor (TLR) signaling that causes NF-κB activation and the production of both inflammatory cytokines and type I interferons. Ngo et al. found that 29% of patients diagnosed with ABC subtype DLBCL had a leucine (CTG) to proline (CCG) exchange at position 265 (L265P) of the myeloid differentiation major response gene 88, which may be another factor which contributes to NF-B hyperactivity. PIM1 expression is correlated with poor prognosis in DLBCL, NOS, and the most common PIM1 mutations identified in patients with poor response to targeted therapy are G28D, L2V, and S97N. Therefore, PIM1 appears as an attractive target in the therapy of hematopoietic neoplasms and as a biomarker of early progression. However, MYD88 L265P and PIM1 p.G28A, p.L184V and p.V197F mutations have not yet been examined together with MYD88 and PIM1 protein expression in cases of large B-cell NHL. In this study, we aimed to we determine the frequency of MYD88 L265P and PIM1 p.G28A, p.L184V, p.V197F mutation, the level of MYD88 and PIM1 immunohistochemical expression and their associations with each other and with clinico-pathological parameters among patients with large B-cell non-Hodgkin's lymphomas in Romania. Therefore, it may be essential to perform a determination of these mutations and, if necessary, to classify tumors in light of the presence of the mutation in lymphomas.

6.4. Results

In the present study, we identified 50 cases of large B-cell non-Hodgkin's lymphomas, which were represented by the types DLBCL (80%), HGBL (8%), THRLBCL (8%) and PCLBCL-LT (4%) . In the case of the cell of origin of DLBCL, the most frequently identified was the GCB subtype (80%). Large B-cell non-Hodgkin's lymphomas affected both sexes equally (M:F–1:1). The average age of the patients was 61.04 years (with extremes between 27-101 years), the majority of patients being over 60 years old (60%). More than half of the cases were identified in the lymph nodes (54%). The other main localizations of large B-cell NHL, according to the analysis of the studied group, are at the level of the gastrointestinal tract (30%) and the CNS (8%). Also, the most frequent localization in the gastrointestinal tract was at the level of the stomach (10%) and the small intestine (10%).
Patients diagnosed with lymphoma had a low ECOG performance status (0-1) at admission in most cases (70%). According to the patient's hospitalization medical records, the most common comorbidities reported were secondary anemia (74%), hypertension (20%), and diabetes mellitus (14%).

The advanced age of the patients, especially those over 60, was associated with the presence of arterial hypertension (p=0.038, respectively p=0.031). An association with the presence of atrial fibrillation in elderly patients was also observed (p=0.016). The presence of dyslipidemia was observed especially in the case of DLBCL, showing significant statistical associations with the female sex (p=0.018). It was noted that hypertension is in close association with hyperuricemia (p=0.020), atrial fibrillation (p<0.001), hematuria (p=0.002), renal failure (p=0.003), upper digestive hemorrhage (p=0.003) and heart failure (p=0.003). <0.001).

Some of the patients had a history of acute infections in 16% of cases (with Klebsiella pneumoniae, Staphylococcus epidermidis, Streptococcus, Bacillus spp., Pseudomonas aeruginosa; Helicobacter Pylori and Candida Albicans) or chronic infections such as hepatitis C virus (HCV, 8%), tuberculosis (TB, 8%), hepatitis B virus (HVB, 4%) or human immunodeficiency virus (HIV, 4%).

Patients with HIV infection were younger than patients without HIV infection (p=0.003). A predominance of male patients was observed in the distribution of history of acute infectious diseases (p=0.007), and HCV was observed mostly in female patients (p=0.038).

Most patients (70%) had a good ECOG performance status (0-1). The main symptoms that led to hospitalization varied, the most frequent being hyperuricemia (16%), hepatosplenomegaly (16%) or hematuria (12%). The presence of hyperuricemia was associated with the presence of concomitant neoplasms (p=0.015).

From a biochemical point of view, the most important change was an increase in serum LDH values (56% of cases). Serum LDH had a mean value of 471.04 IU/L.

In the case of anemic syndrome, the mean value of hemoglobin was 10.93 g/dl (4.90-15.70 g/dl). Regarding the association between laboratory analyses, we observed an increase in serum LDH values at admission that was statistically significantly associated with the presence of anemia (p=0.033), but also with low hemoglobin values (p=0.011). We also observed a statistically significant association between serum LDH values and hemoglobin - the higher the LDH value, the lower the hemoglobin (p=0.001). However, in the case of elevated LDH values, the ECOG performance status was low (0-1), (p=0.033). In the case of hemoglobin, its decrease was also associated with the presence of upper digestive hemorrhage (p=0.021), as well as with other concurrent neoplasms (p=0.046).

A little over half of the cases had nodal localization (54%), being associated with the presence of hepatosplenomegaly (p=0.039). Patients with lymphoma in the small intestine, spleen, brain and testis had a high ECOG performance status (2-4) compared to the other sites (p=0.001).

Depending on the locations of large B-cell lymphoma, testicular or splenic locations were associated with the shortest survival, 7 days (one week) and 49.14 days (7.02 weeks), respectively. Also, a shorter survival was observed in extranodal location of lymphomas compared to nodal location of lymphomas (p = 0.130).

An elevated LDH value was associated with decreased patient survival (p=0.002), 869.34 days (124.19 weeks) versus 2447.10 days (349.58 weeks). An elevated LDH value at admission was an independent negative risk factor for patient survival (HR=3.100, p=0.004).

Ann Arbor staging revealed a slight majority of cases (52%) in the low-grade category (I-II). The same aspect was found and could be transposed in terms of the IPI score, with 64% of cases

being low risk. A low-grade Ann Arbor staging was associated with nodal localizations ($p<0.001$), and a similar pattern was observed in cases with a low IPI score ($p=0.001$). A high IPI score was associated with extranodal sites other than those in the skin or large intestine ($p<0.001$). Also, a high IPI score was noted in cases with elevated serum LDH values ($p=0.001$).
Unfortunately, not all patients benefited from the treatment, only 62% of them, and the preferred treatment protocol was R-CHOP (44%). This treatment scheme was associated especially with a low age of the patients ($p=0.034$), but also with the nodal location of the lymphoma ($p=0.004$). Both R-CHOP and CHOP protocol regimens were associated with good ECOG performance status ($p<0.001$), low Ann Arbor stage ($p=0.005$), and favorable IPI risk score ($p<0.001$).
At the end of this study, only 36% of patients were alive. Median survival was 913.42 days (130.48 weeks). Patients with a good performance status (0-1) had a significantly longer median survival, 2176.49 days (310.92 weeks) versus 54.36 days (7.76 weeks), compared to those with a changed ($p<0.001$). ECOG performance status was an independent risk factor predicting mortality ($HR=9.372$, $p<0.001$).
The most effective treatment for ensuring survival was the use of the CHOP regimen, with a median survival of 2323.32 days (331.90 weeks), while an average of 2278.90 was observed in the R-CHOP regimen days (325.55 weeks), and those who did not undergo chemotherapy had an average of 117.16 days (16.73 weeks), ($p<0.001$). Lack of chemotherapeutic treatment is a risk factor for patient mortality ($HR=6.750$, $p<0.001$).
The IPI score was associated with patient survival, such that those at high risk had a shorter mean of 54.36 days (7.76 weeks) versus 2176.49 days (310.92 weeks) compared to those in the with low risk ($p<0.001$). The high risk represented by the IPI score is a negative risk factor in patient survival ($HR=4.654$, $p<0.001$).
Multivariate analysis of the data shows that the associations between a moderately low performance status (2-4) over 60 years of age and an elevated LDH value with the presence of anemia are risk factors for survival ($HR=7.715$, $p<0.001$, respectively $HR=3.582$, $p=0.020$).
Genetic analysis revealed a mutant status of MYD88 in 8% of cases and of PIM1 in 2% of cases. Among the patients with L265P mutation, there were one male patient and three female patients, with a mean age of 68.5 years, ranging from 54 to 82 years. Three of the patients were older than 60 years. None of the patients with the L265P mutation showed a significant association with clinical parameters of DLBCL, including patient age, gender, tumor location, ECOG performance status, serum LDH level, IPI score, and Ann Arbor stage. All but two of these patients were diagnosed with diffuse large B-cell lymphoma, the non-GCB subtype.
MYD88 was expressed in lymphoid cells in 8 (16%) of 50 cases. Regarding MYD88 immunopositivity, quantification of the reaction revealed a low score (2-4) in 10% of cases and a high score (5-6) in 6% of cases. A high score was associated with HGBL lymphomas, while a low score was correlated with THRLBCL and PCLBC ($p=0.005$). In DLBCL cases, immunopositivity quantified as low and high score is associated with non-GCB origin ($p<0.001$), but not with other clinicopathological parameters. No statistically significant correlation was

observed between MYD88 expression and L265p MYD88 mutation (Spearman ρ = -0.072, p=0.617).
Genetic analysis revealed a mutant status of PIM1 in 2% of cases. PIM1 p.G28A was observed in 1 of 4 THRLBCL cases. PIM1 was expressed in lymphoid cells in 27 (54%) of 50 cases. None of the patients with the PIM1 p.G28A mutation showed a significant association with clinical parameters, including patient age, gender, tumor location, ECOG performance status, serum LDH level, IPI score, and Ann Arbor stage.
Multivariate analysis revealed that the association between a high LDH value at admission and the immunohistochemical expression of PIM1 or the mutant status of the PIM1 gene, represent negative prognostic factors (HR=2.066, p=0.042, respectively HR=3.100, p=0.004).
No statistically significant correlation was observed between PIM1 expression and the PIM1 p.G28A mutation (Spearman ρ = -0.132, p=0.361). In the case of the mutant status, no deaths were noted, these being noted only in the case of the "wild type" status.

6.5. Discussions

In the current study, DLBCL, NOS is the most common subtype of lymphoma in the cohort of patients examined (80%), and the findings were in agreement with data provided by WHO 2022.
Consistent with the findings of previous studies, we noted that half of DLBCL, NOS cases were identified in the lymph nodes (50%). In our research, the most common extranodal locations of large B-cell lymphomas are in the gastrointestinal tract (30%), represented by the stomach (10%), small intestine (10%) and large intestine (6%).
In our study, the mean age of patients diagnosed with DLBCL, NOS is similar to the previous estimate (60.78 years). Limited data are available specifically for HGBL, NOS, but at least it is established that its incidence generally increases with age and, consequently, elderly patients are most often affected. In our study, the mean age of patients diagnosed with HGBL, NOS was below these values (55.25 years), highlighting the predominance of the disease in younger patients. THRLBCL accounts for <10% of all large B-cell lymphomas and preferentially affects middle-aged or elderly adults (age range 18–90 years), rarely affecting children. In our study, the mean age of patients diagnosed with THRLBCL was similar to the values reported in the literature (58 years). PCLBCL-LT usually occurs in elderly patients with a mean age of about 75 years, but in our study, the mean age of PCLBCL-LT patients was higher than these values (84 years).
Our research identified cases where the cell of origin could be identified (80%), with a higher predominance of the GCB subtype (64%) and a lower predominance of the non-GCB subtype (16%). DLBCL, NOS generally occurs slightly more frequently in males compared to females and similarly, in the present study, male patients are most frequently diagnosed with DLBCL, NOS, a fact observed in 52.5% of cases.
No gender predilection was reported in cases diagnosed with NOS high-grade large B-cell lymphoma. In our research, the female sex is most frequently affected (75%). THRLBCL has a slight male predominance with a male:female ratio of 1.7-2.6:1. In contrast, in our study, THRLBCL affects both sexes equally (M:F = 1:1). PCLBCL-LT is more common in female patients, with a female:male ratio of 2:1 to 4:1. In contrast, in our study, PCLBCL-LT affects both sexes equally (M:F = 1:1).

Our research identified that, unfortunately, not all patients benefited from the treatment, only 62% of them, and the preferred regimen was R-CHOP (44%). In our study, the treatment regimen with R-CHOP was especially associated with the reduced age of the patients (p=0.034), but also with the nodal location of the lymphoma (p=0.004). Both R-CHOP and CHOP protocol regimens were associated with good ECOG performance status (p<0.001), low Ann Arbor stage (p=0.005), and favorable IPI risk score (p<0.001). Also, in the present study, the most effective treatment for survival was the use of the CHOP scheme, in its case a mean survival of 2323.32 days (331.90 weeks) was observed, while in the use of the R- scheme CHOP, a mean of 2278.90 days (325.55 weeks) was noted, and those who did not undergo chemotherapy had a mean of 117.16 days (16.73 weeks), (p<0.001). Lack of chemotherapeutic treatment is a risk factor for patient mortality (HR=6.750, p<0.001).

In the present study, regarding patient survival according to serum LDH value, an increased LDH value was associated with reduced patient survival, 869.34 days (124.19 weeks) versus 2447.10 days (349.58 weeks), (p=0.002). A high LDH value at admission was an independent negative risk factor for patient survival (HR=3.100, p=0.004).

In a research study by Prochazka et al., higher uric acid levels were associated with reduced progression-free survival (PFS, hazard ratio (HR)) and overall survival (OS) in the analysis of time to to the univariate event. Hyperuricemia (20%) was one of the most prevalent comorbidities in our study group, although it was not associated with a reduced overall survival rate.

In our study, genetic analysis revealed a MYD88 mutant status in 8% of patients and the L265P mutation was observed in 4 of 40 DLBCL cases (Table 94). Also, none of the patients with the L265P mutation showed a significant association with clinical parameters of DLBCL, including patient age, gender, tumor location, ECOG performance status, LDH level, IPI score, and Ann Arbor stage. Patients with DLBCL, GCB subtype demonstrated a reduced frequency of mutation, while patients with DLBCL, non-GCB subtype showed MYD88 mutant status in 27.8% of cases. These results are consistent with the findings of other studies that the prevalence of MYD88 L265P mutations in DLBCL patients ranged from 6.5 to 19%. In our study, the mutation frequency was similar between GCB and non-GCB subtypes of DLBCL.

Of the four MYD88 L265P mutations, two of them had nodal involvement, while the other two had extranodal involvement, including immune-privileged locations. Patients with extranodal involvement presented in advanced Ann Arbor stage (III-IV). Because MYD88 mutation is present in several B-cell lymphomas, it has been hypothesized that MYD88 mutation represents an early molecular event in lymphomagenesis. A study by Fujiishi et al., the mutation was not detected in a diagnostic biopsy sample of a DLBCL patient, although the post-mortem sample of another DLBCL patient tested positive for the mutation, they hypothesized that the mutation would could be related to a more aggressive phenotype. According to our findings, individuals with the MYD88 L265P mutation were characterized by an advanced stage, which raises the possibility that the mutation is crucial in the development of lymphoma and is related to a worse prognosis for the disease.

According to a study by Kraan et al., MYD88 mutations were relatively uncommon in DLBCL, the activated B-cell (ABC) subtype that develops in the lymph nodes or gut; however, the mutation rate was higher in tumors originating from immune-privileged sites. Also, other studies indicated that MYD88 mutations were more prevalent in primary lymphomas of the central nervous system. In contrast, in our research the MYD88 L265P mutation was detected in half of patients with non-GCB DLBCL either with nodal involvement or with involvement of immune-

privileged sites: the central nervous system (brain). Similarly, only half of patients with DLBCL, the GCB subtype with nodal involvement had the MYD88 L265P mutation.
To the best of our knowledge, only Choi et al. and Caner et al. evaluated MYD88 expression by IHC analysis in DLBCL. Choi et al.'s study did not identify any association between MYD88 expression and clinicopathological variables such as Ann Arbor stage or IPI score. In contrast, the study by Caner et al. found an inverse relationship between MYD88 overexpression and Ann Arbor stage and IPI score respectively. In our research, we also analyzed MYD88 protein expression by IHC analysis in mature B-cell NHL, and similar to the results reported by Choi et al. and Caner et al., respectively, our study failed to reveal the correlation between MYD88 mutation and expression. A potential limitation of our study is that the focus was exclusively on the MYD88 L265P mutation, the most prevalent mutation identified in lymphomas; other MYD88 variants, such as S222R and T294P, could be responsible for altered protein expression. Therefore, more research is needed to understand other genetic and epigenetic changes underlying MYD88 expression identified in more patients diagnosed with B-cell NHL.
According to Zhou et al., PIM1 and MYD88 were highly expressed in the patient cohort and were related to their overall survival time, and high expression of PIM1 or MYD88 was correlated with a high risk score, and high expression of MYD88 was also correlated with elevated serum LDH levels. However, there was no significant correlation with age, patient gender and/or type. Also, the same research report showed that multivariate Cox regression model analysis, including risk score, LDH level, treatment method, and PIM1 and MYD88 expression status, indicated that MYD88 expression status was an independent predictor of OS with a hazard ratio (HR) of 0.004. In accordance with these studies, in our case the multivariate analysis identified that the association between a high LDH value at admission and the immunohistochemical expression of PIM1 or with the mutant status of the PIM1 gene represent negative prognostic factors (HR=2.066, p=0.042, respectively HR=3.100, p=0.004).

6.6. Conclusions

In conclusion, our study identifies that compared to previously reported studies, the incidence of MYD88 L265P and PIM1 p.G28A mutations in DLBCL patients is lower and provides a clear picture of the mutational landscape in DLBCL, which may lead to new insights to treat the condition. Thus, regardless of the MYD88 L265P mutation, MYD88 expression may have significant implications on DLBCL progression, independent of the MYD88 L265P mutation.

For this reason, we suggest that MYD88 expression and the L265P mutation should only be used as prognostic indicators for late-stage disease cases. Regardless of mutation, MYD88 expression does not indicate disease prognosis.

Regarding PIM1, because of the association between high admission serum LDH and PIM1 immunohistochemical expression or PIM1 gene mutant status, we hypothesized that both immunohistochemical expression and PIM1 gene mutation could be used as prognostic factors. prognosis in patients diagnosed with large B-cell lymphoma.

9. The originality of the thesis

9.1. The originality of the thesis

- It is the first study that aims to investigate the concomitant presence of MYD88 and PIM1 oncogenic mutations, both by real-time polymerase chain reaction (RT-PCR) and immunohistochemical evaluation, with possible implications for disease treatment and prognosis among patients with large B-cell non-Hodgkin's lymphomas from the central and south-eastern region of Romania.
- It is the first study that aims to evaluate the expression of the proliferation index Ki67 by means of artificial intelligence (QuPath software), as well as the role of digital pathology in prognostic significance, in patients with non-Hodgkin's large B-cell lymphomas with extranodal - gastrointestinal localization in the Central and Southern-East region of Romania.
- Evaluation of the expression of an original panel of oncogenic genes associated with tumor proliferation and progression with the aid of immunohistochemistry and molecular biology techniques, adapted and optimized to the specific working conditions of the histopathology and molecular biology laboratories within CEDMOG.
- The results obtained from this study complete the existing panel of biomarkers and oncogenic mutations in diagnosis, being useful in detecting the complex histomolecular mechanisms involved in the pathogenesis of large B-cell non-Hodgkin's lymphomas and may influence the final therapeutic management.
- Evaluation of the role of immunodeficiency and antiretroviral therapy in the development of large B-cell non-Hodgkin's lymphomas.
- The data obtained from this scientific work can serve as a reference for future studies in the field of biomarker research and oncogenic mutations or as a basis for the development of new personalized therapies.

9.2. Limitations of the study

Several limitations should be considered when interpreting the results of this study. First of all, the number of evaluated patients is not large enough to provide a complete picture on the variation of gene expression as well as the biomarkers MYD88 and PIM1 respectively on the variability of the expression of the proliferation index Ki67 evaluated by means of digital pathology in patients with non-Hodgkin's lymphomas with large B cell with gastrointestinal localization. So, in order to confirm the results of the study in the population of central and southeastern Romania, additional studies that include more groups of patients are needed to support the findings.
Second of all, all human DNAs that were studied were based on a careful review of the literature, with the suspicion that other DNA species might be more prominently overexpressed or underexpressed.
Third of all, a much longer period of time and additional randomized trials are needed to gradually evaluate the role of immunodeficiency and antiretroviral therapy in the occurrence of this category of lymphomas.

9.3. Future research directions

In the present, the study of genic expression and diagnosis by means of artificial intelligence in large B-cell non-Hodgkin's lymphomas offer promising perspectives and open new therapeutic opportunities globally, and molecular biology and digital pathology are becoming an important part in the diagnosis of this pathology, with an impressive rate of ascent. Thus, it is necessary to develop both a routine screening program and a software specialized in hematopathology, and their implementation both nationally and internationally in the diagnosis of large B-cell non-Hodgkin's lymphomas.

In the future, the discovery and development of specific and sensitive biomarkers or virtual pathology programs in the early diagnosis of large B-cell non-Hodgkin's lymphomas will be a vital objective in personalizing the therapeutic management of the oncological patient.

Another possible direction would be the study and testing of targeted therapies against non-compliant lymphomas, which present the expression of these oncogenic mutations. In this way, the potential of these biomarkers as potential therapeutic targets could be explored.

Another important challenge is the translation of new scientific knowledge into diagnostic, prognostic and therapeutic tools with clinical applicability in the treatment of large B-cell non-Hodgkin's lymphomas.