Molecular and Clinical Analysis of Large B-Cell Lymphomas with *MYC* Gene Involvement: Moving Towards a Molecularly Defined Risk Stratification

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Abstract

Diffuse Large B-cell lymphoma (DLBCL) is a heterogeneous disease, with different biological features and clinical behaviors. High-grade B-cell lymphomas (HGBCL) with *MYC* and *BCL2* and/or *BCL6* rearrangement represents a separate entity with a lower response to R-CHOP like regimens and poor outcome. A more accurate risk stratification of DLBCL/HGBCL harboring *MYC* alterations may provide further prognostic information for an optimal choice of therapy.

Major factors impacting risk stratification are:

- **Characterization of *MYC* translocation partners**
- **Presence of *TP53* gene alterations**
- **Determination of Cell of Origin (COO)**

This is a retrospective study on a multicentric cohort of DLBCL/HGBCL with *MYC* gene involvement (*MYC* rearrangement and/or *MYC* anomalies identified by Fluorescent *In Situ* Hybridization, FISH).

The following analyses were performed:

- **Immunohistochemistry**
- **FISH**
- **Targeted Next Generation Sequencing (NGS)**
- **Gene Expression Profiling Study (GEP)**

Biological findings were correlated with baseline clinical data and survival outcomes.

42 patients were included in the study (38/42 with DLBCL and 4/42 with HGBCL). All patients received first line treatment with R-CHOP like regimens. Median follow-up was 36 months. Median age was 65 years; 15/42 (36%) patients were low/intermediate International Prognostic Index (IPI) and 26/42 (62%) were intermediate-high/high IPI risk (1/42 data not available).

MYC increased copy numbers were detected in 8/42 (19%) patients, whereas *MYC* rearrangements (MYC-r) in 34/42 (81%). Of the MYC-r patients, 31/34 patients had sufficient material for subsequent analyses. *MYC* rearrangement with an *IG* gene was observed in 19/31 (61%) patients. *BCL2* and *BCL6* rearrangements were reported in 20/39 (51%) and 14/39 (36%) cases, respectively. The co-occurrence of *MYC* and *BCL2* rearrangements was detected in 12/31 (39%) patients, *MYC* and *BCL6* in 6/31 (19%) patients and 4/31 (13%) patients were altered for all three genes. *A TP53* deletion was present in 7/40 (18%) patients for whom the analysis was available.

COO was Germinal Centre B-Cell (GCB) subtype in 31/40 (78%) patients, Activated B-Cell (ABC) in 6/40 (15%) and unclassified in 3/40 (7%).

The most prevalent pathogenic variants detected by NGS (i.e. detected in >15% patients) were ***TP53*** (33%), ***CREBBP*** (31%), ***MYC*** (31%), ***BCL2*** (19%), ***TNFRS14*** (19%), ***CIC*** (17%), ***EZH2*** (17%) and ***NF1*** (17%). Subsequently, NGS data were applied to the LymphGen algorithm with success in 38 out of 42 cases (90%). The subgroups were distributed as follows: **EZB** (13/38; 34%), **MCD** (6/38; 16%), **ST2** (2/38; 5%), **N1** (1/38; 3%), **BN2/N1** (1/38; 3%), **BN2** (1/38; 3%), and **OTHER** (14/38; 37%).

The three-year overall survival (OS) rate was 52% in all patients with *MYC-r* and 75% in patients with *MYC* gain (p=0.19). As expected, patients with *MYC* and *BCL2* and/or *BCL6* rearrangements showed a trend to worse OS (3-year OS rate 46%) as compared with patients with *MYC-r* only or with *MYC* gain (3-year OS rate 75% in both groups, p= 0.39). Patients with *MYC-r* and *TP53* deletion showed a significant inferior OS (3-year OS 20%) as compared to patients without *TP53* deletion and with *MYC-r* (3-year OS 59%) or *MYC* gain (3-year OS 75%) (p-value = 0.047). Interestingly, cases with *MYC* abnormality and a *TP53* deletion and/or *TP53* mutation showed high-risk disease, as demonstrated by the reduced 3year OS (35%) compared to the other cases (73%) with a statistically significant difference (p=0.016).

With the limitations of the small sample size and the retrospective nature of the study, our findings underline the adverse prognostic impact of *TP53* alterations (deletions and mutations) on OS in a cohort of DLBCL/HGBCL patients harboring *MYC* alterations (structural and/or numerical). The adverse clinical impact of the co-occurrence of *MYC* and/or *BCL2*/*BCL6* rearrangements was confirmed. Evaluation of *TP53* status by FISH and sequencing should be included in the standard diagnostic work-up of DLBCL/HGBCL patients to further refine the prognosis as *TP53* status could potentially help tailor treatment decisions, by considering novel immunotherapies earlier in the disease course in dedicated clinical studies.

Introduction

Lymphoid Neoplasms

Lymphoid neoplasms are malignant tumors, originating from the clonal neoplastic transformation of immunocytes (B cells, T cells, or Natural Killer (NK) cells) at various stages of their maturation and differentiation. These tumours can present in different ways: localizing within the lymphatic system, in lymph nodes, spleen, thymus, and bone marrow, or extending beyond these sites, infiltrating other organs and tissues.

In recent years, the introduction of advanced diagnostic technologies, such as Next-Generation Sequencing (NGS) and Gene Expression Profiling (GEP), has greatly enhanced the insight on neoplastic malignancies, enabling a more comprehensive molecular profiling. These technologies, alongside traditional methods like immunohistochemistry (IHC) and Fluorescent In Situ Hybridization (FISH), are facilitating the identification of molecular subtypes of lymphoid neoplasms.

Recently, as international standard for diagnosis, two distinct classifying proposals of lymphoid neoplasms appeared: The 5th edition of the *Haematolymphoid Tumours* classification by World Health Organization (WHO) (WHO-HAEM5) and the International Consensus Classification (ICC) [1], [2]. Both integrate these improvements, introducing new subgroups of lymphomas with distinct genetic and molecular. This paradigm shift aims first at improving the diagnostic accuracy but also at leading to a more targeted treatment strategies, thus ensuring that patients receive the most appropriate therapeutic strategies based on the specific biology of their disease.

B-cell Lymphoid Neoplasms

B-cell lymphoid neoplasms arise from B cells at various stages of maturation. During the entire process of cellular differentiation, these neoplasms may emerge due to genetic/molecular events that trigger transformation and neoplastic progression. The physiological differentiation of B cells starts in the bone marrow, where B lymphoblasts undergo *VDJ* rearrangement at the immunoglobulin heavy chain (*IGH*) locus, maturing into *naïve* B cells. These immature B cells may localize in the mantle zone of the primary follicle or circulate in a quiescence state.

Upon encountering an antigen, activated *naïve* B cells migrate to the centre of a primary follicle, forming a germinal centre (GC) alongside follicular dendritic cells. Here, they

undergo profound transformation, differentiating into memory B cells or mature plasma cells, secreting antibodies. This transformation involves complex genetic remodelling processes, including Somatic Hypermutation (SHM) and Class Switch Recombination (CSR), which enable the production of antibodies that are best suited to target specific antigens.

Of note, the finely regulated genetic mechanisms responsible for developing high-affinity immunoglobulin of various isotype classes can also play a role in neoplastic transformation. In fact, the B cells within the GC represent the normal counterpart for most mature B-cell lymphomas. The GC consists of a dark zone at the base, where *naïve* B cells actively proliferate and undergo SHM, transforming into centroblasts. The **centroblasts**, based on the analogy of the mutational/transcriptional profiles, represent the normal counterparts of Burkitt lymphoma (BL) and of subgroup of highly proliferative and clinically aggressive Diffuse Large B-Cell Lymphomas (DLBCL).

Subsequently, B cells migrate toward the inner light zone of the GC, where they are selected based on their affinity for the antigen, differentiating into **centrocytes**. The centrocytes and centroblasts, in different proportion, represents the normal counterpart of Follicular Lymphoma (FL), a lymphoid neoplasm, often clinically indolent, that can progress to aggressive forms of DLBCL or High-Grade B-Cell Lymphoma (HGBCL). Additionally, a subset of DLBCL arises from post-germinal centre B cells destined to differentiate into **plasmablasts**, sharing a similar molecular expression profile.

Once centrocytes complete their maturation process, they enter circulation and can give rise to **memory B cells**. These cells, typically localizing in the marginal zones of lymph nodes, the spleen, and mucosa-associated lymphoid tissue (MALT), represent the normal counterpart of nodal marginal zone lymphomas and the extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue. Finally, centrocytes may differentiate into **mature plasma cells,** secreting antibodies, which, thanks to a phenomenon known as “homing,” return to the bone marrow. These cells represent the normal counterpart of Multiple Myeloma (MM) cells [3], [4]. In the figure, a schematic representation of the different maturation steps and the correlation of them with the main B neoplasms mentioned above (Fig 1).

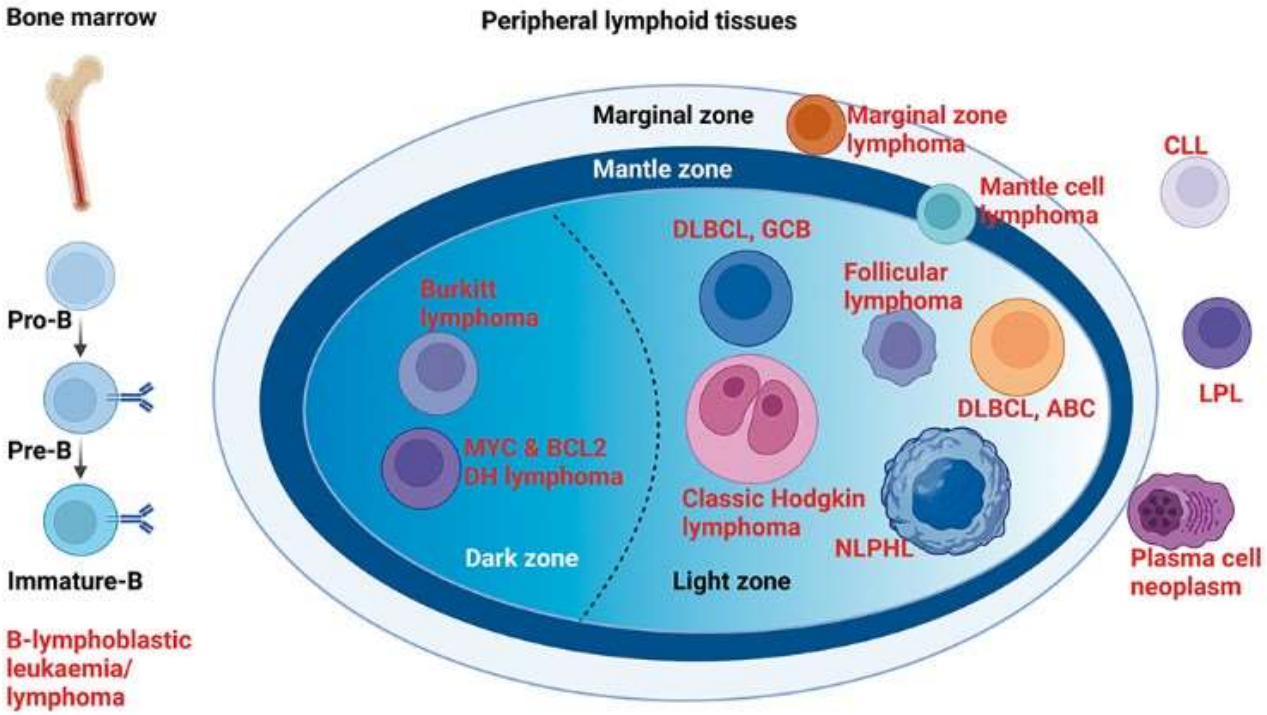


Fig. 1 Schematic representation of the different maturation stages and their correlation with the main B-cell neoplasms [5].

Diffuse Large B-cell lymphoma

The family of large B-cell lymphomas encompasses a range of tumors with diverse morphologies, genetic characteristics, and clinical behaviors. It accounts for approximately 30% of Non-Hodgkin lymphomas (NHL), with one of the highest incidences in the world, with 73,652 new cases in the United States and 72,035 in Western Europe in 2020 [6]. DLBCL can arise *de novo* or result from the progression of indolent and less aggressive lymphomas, such as lymphocytic lymphoma, follicular lymphoma, and marginal zone lymphoma. Under a clinical perspective, in 70% of cases the disease manifests itself at the lymph node level but, albeit in a smaller percentage, it can affect the gastrointestinal tract, the mediastinum, the salivary glands, the thyroid and the skin, brain and bone tissue. According to WHO-HAEM5 classification, large B-cell lymphomas have been classified into distinct morphological variants, molecular subgroups and distinct nosological entities based on morphology, biological and clinical characteristics [1]. ICC classification emphasizes that DLBCL, NOS is not a single disease but a collection of morphological, genetically, and clinically different diseases, but argues the role of morphologic variants (centroblastic, immunoblastic, and anaplastic)

and phenotypic variants should be deemphasized, highlighting that a combination of COO and molecular subclassification may provide more precise patient stratification for developing future clinical trials.

In figure 2, an algorithm is shown, by Alaggio et al.[1], for the classification of aggressive B-cell lymphomas based on the modifications made in the latest edition of the WHO.

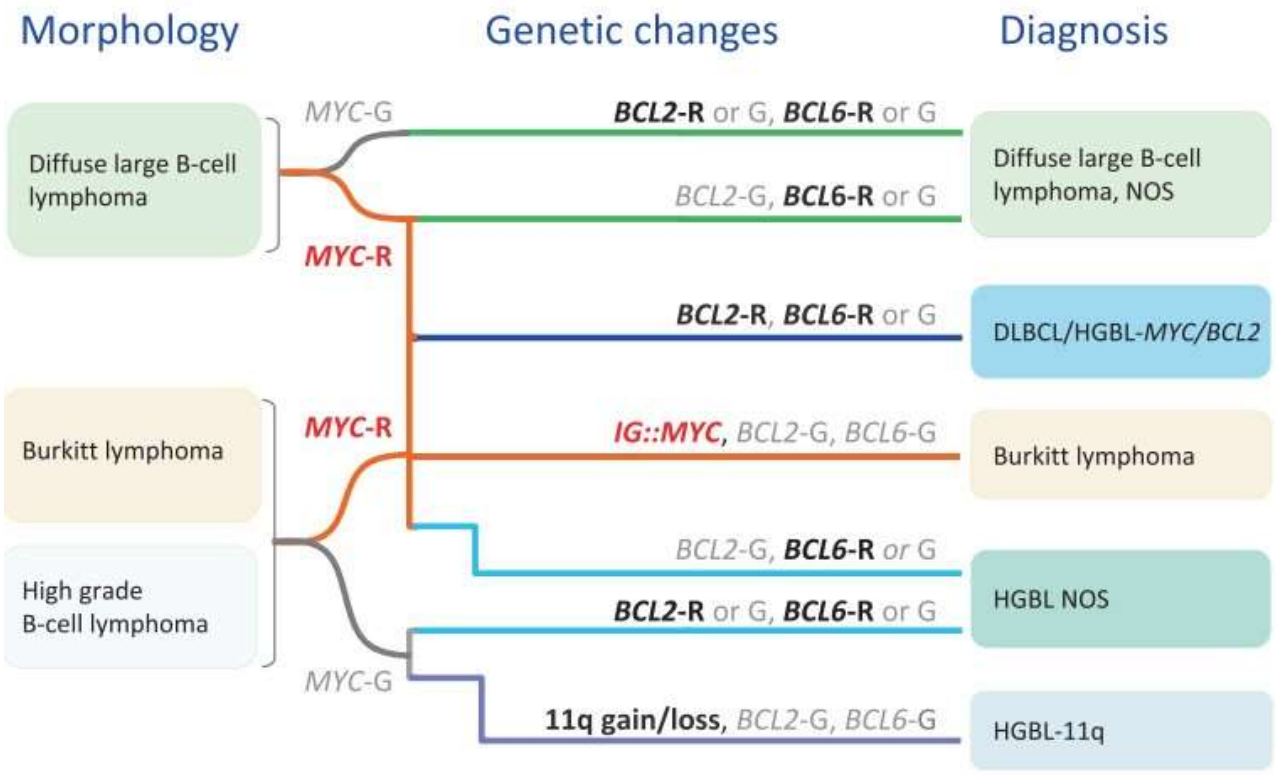


Fig. 2 adapted from R. Alaggio *et al.*, "The 5th edition of the World Health Organization Classification of Haematolymphoid Tumours: Lymphoid Neoplasms," *Leukemia*, vol. 36, no. 7, pp. 1720–1748, Jul. 2022, doi: 10.1038/s41375-022-01620-2.

Nowadays, from a practical point of view, most cases fall into the group of Diffuse Large B-cell Lymphomas, not otherwise specified (DLBCL-NOS), i.e. a group of large-cell B-lymphomas with diffuse growth patterns without specific genetic-molecular characteristics, allowing them to be included in a specific category.

Thus, DLBCL-NOS, defined by its morphology and mature B-cell phenotype, is classified only when the lymphoma does not meet the criteria for a more specific subtype.

The neoplastic cells have a specific immunophenotypic profile and express B cell marker, such as CD19, CD20, CD22, CD79a and PAX5. The CD20 marker, in addition to a diagnostic role, has an important predictive significance as a molecular target of the monoclonal antibody Rituximab, which in combination with chemotherapy significantly improves the

prognosis of patients [6]. The proliferation index, assessed by the expression of the Ki-67 protein, tends to be high and can be greater than 90%. Several new biological markers have been identified as complementary prognostic indicators to the currently used International Prognostic Index (IPI). According to the indications of the latest edition of the WHO, together with the morphological analysis and the evaluation of the proliferation index, for a proper diagnostic classification of lymphomas, complementary analyses are necessary aimed at identifying:

- **Cell of Origin (COO)**; assessed by GEP and surrogate by IHC.
- **Protein co-expression of Myc and Bcl2**; assessed by IHC.
- **Rearrangements in the *MYC*, *BCL2* and *BCL6* genes**; assessed by FISH.

In addition, different molecular signatures have been proposed, although their integration in diagnostic workflow is not completely established [7], [8], [9], [10], [11].

Cell of Origin (COO) Classification in DLBCL

Building upon the study by Alizadeh and colleagues, B-cell neoplasms have begun to be investigated from a more molecular perspective [8]. The authors conducted a GEP study on a large cohort of patients with DLBCL and discovered that there was no single expression profile within these tumors. Instead, they found that genes involved in various biological processes exhibited distinct activation patterns. This observation has paved the way for a deeper understanding of the molecular basis of DLBCL and highlights the complexity and heterogeneity of these neoplasms at a genetic level.

Notably, the study revealed a clear distinction in gene expression between those genes that are constitutively active in the GC and those specific to activated B cells in the post-germinal centre phase. This differentiation enabled researchers to categorize the clinically heterogeneous group DLBCL into two distinct nosological entities. Such insights not only enhance our understanding of DLBCL but also underscore the potential for more targeted therapeutic approaches based on the underlying molecular characteristics.

The COO classification is a crucial aspect of understanding DLBCL and plays a significant role in prognosis and treatment strategies. As previously reported, DLBCL is primarily categorized into two main subtypes based on the COO: Germinal Centre B-Cell (GCB) and Activated B-Cell (ABC) subtypes. This classification is based on gene expression profiling that reflects the cellular lineage from which the lymphoma originates.

- **Germinal Centre B-Cell Subtype (DLBCL-GCB):** The GCB subtype of DLBCL is thought to arise from B-cells that are in the germinal centre stage of development, where B-cells undergo maturation, selection, and affinity maturation in response to antigen exposure. DLBCL, GCB type is generally associated with a more favourable prognosis and a better response to standard immunochemotherapy regimens, such as R-CHOP [8]. Patients with this subtype often exhibit characteristics such as a lower International Prognostic Index (IPI) score and a higher likelihood of achieving complete remission.
- **Activated B-Cell (ABC) Subtype (DLBCL-ABC):** In contrast, the ABC subtype is derived from B-cells that are in an activated state, often reflecting a response to chronic inflammation or antigen stimulation. This subtype is associated with a poorer prognosis and is more resistant to conventional therapies [8]. DLBCL, ABC subtype frequently exhibits alterations in signalling pathways, such as NF-kB and BCR signalling, which contribute to its aggressive behaviour. Patients with this subtype tend to present with higher IPI scores and are less likely to achieve durable responses to treatment.

Finally, there is a subset of lymphomas that cannot be classified based on the GEP profile and therefore remains "unclassified" (U-DLBCL).

The relative frequency of the GCB, ABC and U-DLBCL subgroups varies according to the geographical location, the average age of the patients and the methodology used, with an average frequency of about 60%, 40% and 10-15% respectively [12].

Lymphomas, classified in this way, differ in chromosomal structure, the type of chromosomal aberrations, mutational status and clinical course. The accuracy of the definition of the cell of origin is therefore an important diagnostic and prognostic factor for DLBCLs, useful for the most appropriate therapeutic choice.

GEP profiles, strongly supported by the WHO, are adopted as a classification criterion for DLBCL based on the cell of origin. The WHO recommends the definition of COO with molecular methods (GEP) or, in case more advanced technologies are not available, with IHC algorithm.

Figure 3 shows the heatmap, taken from the original paper, in which the gene expression of DLBCL lymphomas is compared with its normal counterparts.

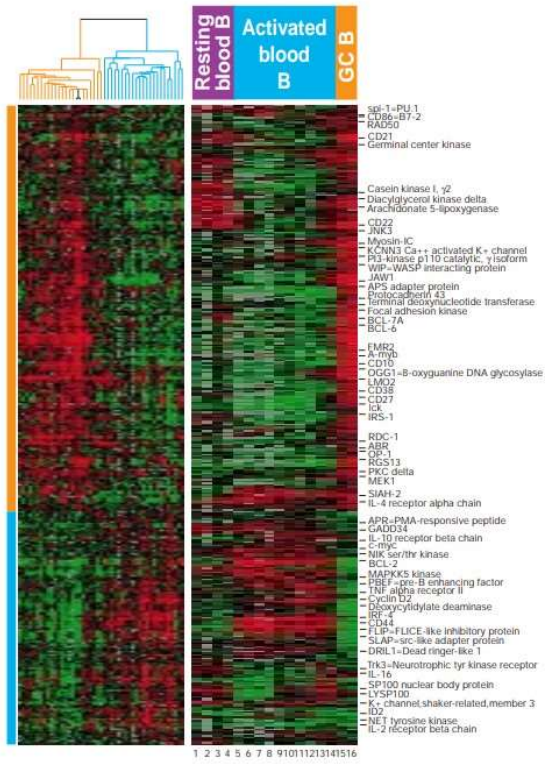


Fig.3 Alizadeh AA, et al. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. Nature. 2000 Feb 3;403(6769):503-11. doi: 10.1038/35000501. PMID: 10676951.

"Double Expressor" Lymphomas

About 30% of DLBCL, NOS is defined as Double Expressor (DE), based on the co-expression of the Myc and Bcl2 proteins evaluated by IHC; in most cases, the cell of origin is a B cell activated post germinal centre (ABC-DLBCL). The concomitant Myc and Bcl2 expression correlates but is not necessarily associated with the presence of the rearrangement of the respective genes. The DE percentage reported in lymphomas with *MYC* and *BCL2* rearrangement is about 75%, while in lymphomas with *MYC* and *BCL6* gene rearrangement it is 53%. Thus, Double protein expression cannot therefore be an unequivocal indicator of the presence of *MYC* and *BCL2* gene rearrangements. The increase in protein expression in cases without gene rearrangement is likely attributable to many mechanisms like increase in the number of copies of the gene or to gene amplification or to post-transcriptional events. The evaluation of protein expression in clinical practice is important for prognostic purposes. Studies in the literature report a worse prognosis in DE lymphomas than those that do not express Myc and Bcl2, although more favourable than in lymphomas with gene rearrangement [13].

BCL2, BCL6 and MYC

Regarding the gene and chromosomal structure in DLBCLs, 30% of cases present with the rearrangement of the *BCL6* gene, 20-30% of the *BCL2* gene and 8-14% of cases present with translocations involving the *MYC* gene [1]. With few exceptions, the B-cell

lymphoma translocations do not generate fusion genes and chimeric protein active oncogenes (typical of myeloid neoplasms) but determine the juxtaposition of an oncogene to the heterologous promoter or enhancer (generally of an immunoglobulin locus), with the consequent altered or ectopic expression of the oncogene itself [3].

The ***BCL2* gene** (18q21) encodes a mitochondrial membrane protein with anti-apoptotic function. The chromosomal translocation t(14; 18)(q32; q21), determines the placement of the *BCL2* gene driven by the *GHH* gene enhancer, encoding the immunoglobulin heavy chain. Consequently, the *BCL2* gene is constitutively active. The t(14; 18)(q32; q21) is the most common translocation in GCB-DLBCLs, with a frequency of 30-40%; however, it is not specific to DLBCL, as it is found in more than 80% of follicular lymphomas, some of which transform in GCB-DLBCL [3][10]. Uncommon variants are the translocations t(2; 18)(p12; Q21) and T(18; 22)(q21; q11), juxtaposing the *BCL2* gene to the light chain loci of the *IGK* and *IGL* immunoglobulins respectively [13]. Finally, the translocation t(14; 18)(q32; q21) derives from an altered mechanism of *VDJ* recombination. *BCL2* chromosomal translocations appear early during the development of B cells, at the stage of B lymphocyte precursors in the bone marrow. However, altered oncogene manifest itself in more advanced stages of lymphomagenesis when the altered lymphocyte enters the GC following antigenic stimulation. In this environment, apoptosis is the main selection mechanism to produce antibodies specific to a particular antigen. The deregulation of *BCL2* alone is not sufficient for complete neoplastic transformation, despite providing an advantage in survival with the accumulation of abnormal cells. Consequently, additional genetic-molecular alterations are necessary for the acquisition of a malignant phenotype. One of the most recurrent secondary genetic events is the duplication of derivative chromosome 18, and in the later stages of evolution, the acquisition of the translocation t(8;14)(q24;q32) and deletion 17p, which represent indicators of particularly unfavourable prognostic significance and resistance to therapy [13].

The ***MYC* gene** (8q24) acts as a transcription factor regulating pathways including cell proliferation and growth, DNA replication, apoptosis, and differentiation, making it a powerful oncogene upon dysregulation. In this regard, genetic mechanisms that alter its copy number or structure can contribute to tumorigenesis [14]. *MYC* translocations are present in more than 90% of Burkitt lymphoma cases, but can be found, albeit in a smaller percentage, in other B-cell lymphoid neoplasms such as FL, DLBCL (especially

GCB-DLBCL) and high-grade B cell lymphomas. In Burkitt lymphoma, *MYC* rearrangements always have an *IG* gene as a translocation partner gene, most frequently the *IGH* gene, and are considered an event promoting oncogenesis in a framework of low karyotype complexity [14]. In contrast, *MYC* translocations in other mature B-cell malignancies frequently involve non-IG partner genes and tend to be found in the context of complex karyotypes, often in association with t(14; 18)((Q24; Q32). In fact, these are chromosomal aberrations that appear in the more advanced stages of neoplastic transformation, rather than in the initial stages of the disease. The group of MYC-IG rearrangements includes the chromosomal translocation t(8; 14)(q24; q32) and the less frequent variants t(2; 8)(p12; Q24) and T(8; 22)(q24; q11). These rearrangements involve a juxtaposition of the *MYC* gene with the *IGH*, *IGK* and *IGL* gene enhancers, respectively. High levels of MYC mRNA, due to constitutively active transcription driven by the *IG* gene promoter, promote cell cycle entry and increased proliferation [14], [15]. About 65% of cases of DLBCL with MYC rearrangement have *IG* as partner gene, in most cases *IGH* [12]. Regarding non-IG partner genes, many are reported in the literature, whit the most frequent are listed below.

- *PAX5*
- *BCL6*
- *BCL11A*
- *IKZF1*
- *BTG1*

Notably, not all *MYC* translocations have the same prognostic value: studies in the literature have shown that in patients with DLBCL/HGBCL with rearrangement of *MYC* and *BCL2* and/or *BCL6* treated with standard chemotherapy protocols, the prognosis is worse when the partner gene of the *MYC* rearrangement is an immunoglobulin gene [16], [17]. Building on previous research, Rosenwald et al. showed that in a large cohort of DLBCL patients treated with R-CHOP, having DHL only indicated a worse prognosis when the MYC translocation partner was an *IG* locus [18]. This emphasizes that the specific translocation partner plays a crucial role in prognosis, which should be considered in treatment planning.

The ***BCL6* gene** (3q27) contributes to the regulation of B cell development and differentiation in the GC, and gene rearrangement is a relevant factor in the initiation

and progression of lymphoma. Abnormalities, including translocations involving the *BCL6* gene, occur in approximately 15% of FLs and 30% of DLBCL, particularly in the ABC-DLBCL subgroup. Similarly to *BCL2* and *MYC* genes, the most frequent chromosomal translocation is t(3;14)(q27;q32), which results in the rearrangement of the entire coding sequence of *BCL6* under the control of the *IGH* gene enhancer. In addition, multiple non-IG partner genes may also be implicated, whose expression is physiologically elevated throughout the B cell differentiation process, including post-germinal centre stages. Regardless of the fusion partner, *BCL6* translocations result in gene overexpression, even when the B cell, having left the GC, has a high affinity for the antigen. In this phase normally *BCL6* expression is normally silenced.

High-Grade B-Cell Lymphoma (HGBCL)

High-grade B-cell lymphomas (HGBCL) represent a subgroup of mature large B-cell neoplasms characterized by particularly aggressive clinical behaviour [12]. These lymphomas exhibit a diffuse growth pattern with few small lymphocytic elements. The shape and size of the nuclei are variable but typically range from three to four times larger than normal lymphocytes. The proliferation index is generally extremely high. The average age of onset is between 60 and 70 years, with most patients presenting at an advanced clinical stage (IV) and an unfavourable International Prognostic Index (IPI). The median overall survival (OS) for patients with HGBCL ranges from 4.5 to 18.5 months, and most of these patients are refractory to standard therapies, necessitating more intensive treatment regimens. Notably, 30% of HGBCL cases evolve from a previous B-cell non-Hodgkin lymphoma, such as FL or DLBCL. According to the WHO-HAEM5 classification, high-grade B-cell lymphomas are categorized into two distinct entities: DLBCL/HGBCL with *MYC* rearrangement and *BCL2*, and HGBCL not otherwise specified (NOS). WHO-HAEM5 specifically excluded DLBCL/HGBCL with concurrent *MYC* and *BCL6* rearrangements, reclassifying them into the DLBCL or HGBCL-NOS categories based on morphology. The ICC takes a slightly different approach, recognizing the two WHO-HAEM5 categories, but adds HGBCL with concurrent *MYC* and *BCL6* rearrangements as a provisional entity.

Diffuse large B-cell lymphoma / high-grade B-cell lymphoma with MYC and BCL2 rearrangements

Diffuse large B-cell lymphoma/high-grade B-cell lymphoma with *MYC* and *BCL2* rearrangements (DLBCL/HGBCL-*MYC*/*BCL2*) is an aggressive mature B-cell lymphoma with structural chromosomal aberrations with breakpoints at both *MYC* and *BCL2* loci.

In this diagnostic category, three different subtypes are included.

- **DLBCL/HGBCL-*MYC/BCL2* without *BCL6* rearrangement**; previously classified as "Double Hit" (DH).
- **DLBCL/HGBCL-*MYC/BCL2* with *BCL6* rearrangement**; previously classified as "Triple Hit" (TH).
- **DLBCL/HGBCL-*MYC/BCL2* (with or without *BCL6* rearrangement) with TdT expression**.

DH lymphomas with *MYC* and *BCL2* rearrangement (DH-MYC/BCL2) typically originate from the GC. Identifying the partner gene involved in the *MYC* translocation is critical, similarly to DLBCL, not all rearrangements carry the same prognostic impact. In about a half of DH-*MYC*/*BCL2* lymphomas, *MYC* rearranged with immunoglobulin genes, with *IGH* as the partner gene in 80% of cases, followed by *IGL* and *IGK*. Studies indicate that *MYC* rearrangement with an immunoglobulin gene (MYC-IG) is associated with a worse prognosis compared to rearrangement with a non-immunoglobulin partner (MYC-non-IG). In patients with DH/TH lymphomas, the synergic effect between the anti-apoptotic effects caused by *BCL2* activation and the genomic instability induced by *MYC* promotes oncogenesis and tumor progression, which is exacerbated by increasing cytogenetic complexity. DH-*MYC*/*BCL2* lymphomas frequently exhibit multiple chromosomal aberrations, both structural and numerical, including polysomy of chromosomes such as 7, 8, 11, 12, 18, 20, and X, along with the loss of chromosomal regions 3q27-29, 6q, and 15q26. Chromosomal and genomic instability fosters the accumulation of further chromosomal aberrations and mutations, contributing to the highly aggressive clinical behaviour of the disease and an average survival of 8 to 19 months.

In contrast, **DLBCL/HGBCL with *MYC* and *BCL6* translocations**, but lacking *BCL2* translocation, diverges from DLBCL/HGBCL-*MYC*/*BCL2* in patterns of presentation, frequent non-germinal-center B-cell immunohistochemistry phenotypes, and activated

B-cell–like gene expression profiles [19]. These are aggressive lymphomas with frequent extranodal involvement. The frequency of MYC-IG and MYC-non-IG translocations is comparable to that observed in DH-MYC/BCL2 lymphomas. From a cytogenetic point of view, they have fewer complex karyotypes than DH-MYC/BCL2 lymphomas, while at the molecular level the mutational status is comparable in the two subgroups.

As already said, these cases, in the latest WHO edition, are excluded from the DLBCL/HGBCL-*MYC*/*BCL2* entity and are now included in DLBCL-NOS or HGBCL-NOS (discussed later in the following sections) [12].

Approximately 10-15% of DLBCL/HGBCL-*MYC*/*BCL2* cases exhibit synchronous rearrangement of the three genes *MYC*, *BCL2*, and *BCL6* (previously classified as TH). From a demographic, clinical, and morphological standpoint, these cases share similarities with DH-MYC/BCL2 lymphomas, with extra nodal involvement at the bone marrow level occurring in more than half of affected patients. Most cases present with advanced-stage disease at diagnosis, and about a quarter have a history of prior B-cell lymphoma, predominantly FL. In terms of immunophenotypic profile, nearly all cases show a COO resembling germinal centre B-cells, and 70% demonstrate concurrent expression of Myc and Bcl2 proteins (DE lymphoma). TH lymphomas tend to exhibit a highly complex karyotype. MYC-IG and MYC-non-IG rearrangements are present with approximately equal frequency, with survival rates being lower in cases where *MYC* rearranges with an immunoglobulin gene compared to those with MYC-non-IG translocations (e.g., PAX5, BCL6, and IKAROS). The median OS for TH lymphomas is around 18 months.

HGBCL, NOS (High-grade B-cell lymphoma, Not Otherwise Specified)

High-grade B-cell lymphoma (HGBCL) NOS represents a heterogeneous type of aggressive mature B-cell lymphoma composed of medium-sized or blastoid cells that does not fulfil the diagnostic criteria for other defined lymphoma entities.

Thus, this category includes mature B-cell neoplasms that exhibit aggressive clinical behaviour but lack the genetic characteristics of Double Hit (DH) or Triple Hit (TH) lymphomas, specifically the *MYC*, *BCL2*, and/or *BCL6* rearrangements. These lymphomas do not fall under the categories of DLBCL or BL, although they share some morphological, immunophenotypic, and genetic features with these entities.

From a histopathological perspective, HGBCL-NOS lymphomas exhibit a blastoid morphology and significant immunophenotypic heterogeneity (Fig. 4). Most of these lymphomas express the Bcl6 protein, while the expression of CD10 and IRF4/MUM1 is variable, complicating the determination of the COO. The proliferation index, as assessed by Ki-67, and the protein expression of Myc also show variability.

Partly due to the small sample size, lliterature on the cytogenetic and molecular characteristics of HGBCL, NOS is limited. Approximately 20-35% of cases exhibit a rearrangement of the *MYC* gene (Single Hit, SH), with or without an increased copy number of *MYC* and, more rarely, *BCL2*. Conversely, some cases have been reported with a rearrangement of *BCL2* and an increased copy number of *MYC*. The prognosis for patients with HGBCL, NOS is unfavourable, although it is somewhat better than that for patients with DH HGBCL.

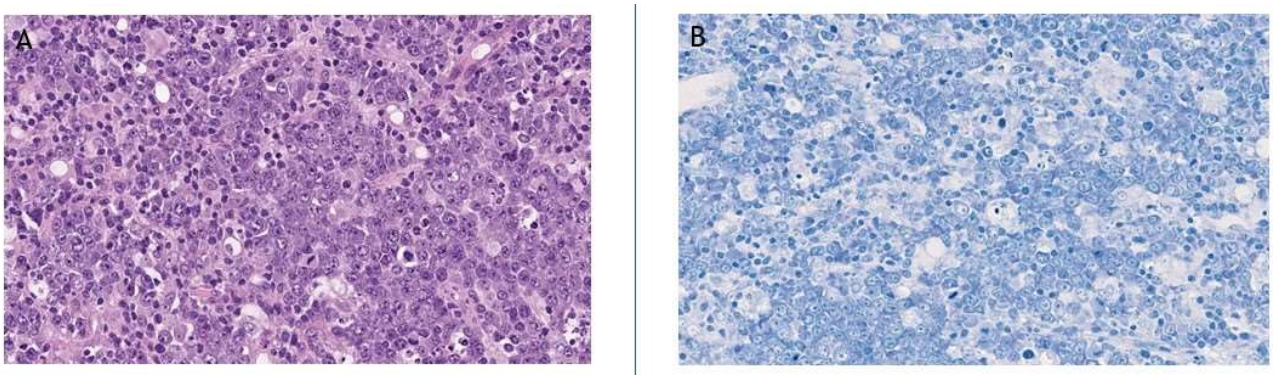


Fig. 4 HGBCL, NOS: Morphology intermediate between DLBCL and BL, characterized by a diffuse proliferation of medium-sized to large cells with a few admixed small lymphocytes. Cellular morphology is variable, exhibiting more variation in nuclear size and nucleolar content than generally acceptable for Burkitt lymphoma. A: Hematoxylin and eosin, B: Giemsa.

High-Grade B-Cell Lymphoma with MYC Rearrangement (Single Hit)

The presence of *MYC* rearrangement as the sole translocation is described in a subset of DLBCL. According to literature data, it occurs in approximately 34% of cases. In some cases, *MYC* rearrangement is associated with the presence of a *BCL2* or *BCL6* copy number gain. *MYC* gene rearrangement is more frequently observed in DLBCL cases originating from the GC. In addition, cases of HGBCL with *MYC* translocations are also reported (8–58% of cases)[20]. These lymphomas are commonly referred to as Single Hit (SH) lymphomas and generally have a worse prognosis compared to those without *MYC* gene rearrangements. The association of *MYC* rearrangement with increased copy number of *BCL2* appears to negatively impact prognosis, although further studies are

needed to clarify this relationship, considering the paucity of available cases with these specific characteristics.

High-Grade B-Cell Lymphoma with Numerical Alterations of MYC, BCL2, and BCL6

A subgroup of HGBCL exhibits numerical alterations of *MYC*, *BCL2*, and *BCL6* that are not associated with the classic translocations characterizing the so-called DH and TH lymphomas. The prognostic significance of changes in the copy number of the *MYC* gene, including gains, duplications, and amplifications, has primarily been studied in DLBCL. Several studies have demonstrated a worse prognosis in DLBCL with gene amplification or variations in the copy number of MYC, particularly when the *MYC* copy number exceeds 4. While the prognostic impact of increased copy numbers of *BCL2* and *BCL6* remains controversial, some studies have reported unfavourable prognoses in patients with DLBCL or HGBCL who have extra copies of *BCL2* or *BCL6*. In a study by Huang et al., which analysed 108 cases of lymphoma with extra copies of *MYC*, *BCL2*, or *BCL6*, it was found that patients with gene rearrangements or increased copy numbers of *MYC* and *BCL2* had lower OS compared to those with a normal gene configuration. In contrast, no significant differences were noted between patients with increased copy numbers of *BCL6* versus those with a normal gene copy number; however, patients with extra copies of *BCL6* showed better prognoses than those with *BCL6* gene rearrangements [21].

TP53

TP53 alterations, particularly mutations and deletions, are closely linked to poor prognosis and resistance to therapy in several types of cancer, including chronic lymphocytic leukemia (CLL), small lymphocytic lymphoma (SLL), and mantle cell lymphoma. *TP53* mutations disrupt the normal activity of the p53 protein, which plays a key role in regulating apoptosis and the cellular response to DNA damage [22]. This leads to increased cell proliferation and heightens genomic instability.

These mutations interfere with apoptosis, promote unchecked tumor growth, and increase genomic instability, complicating the treatment process. Approximately 20-25% of DLBCL cases carry *TP53* mutations, with similar frequencies observed in both GCB and ABC subtypes.

Several studies have demonstrated the detrimental effects of *TP53* mutations in DLBCL. For instance, Xu-Monette et al. reported that, in DLBCL patients treated with R-CHOP

therapy, *TP53* mutations are a significant independent marker of poor prognosis, influencing both OS and progression-free survival (PFS) [23].

In HGBCL, *TP53* mutations occur even more frequently, particularly in the double-hit *MYC/BCL2* translocation subgroup (DH-MYC/BCL2), where the mutation rate is 38% [22]. Conversely, these mutations are less common in cases involving *MYC/BCL6* translocations.

Specifically, the prognostic significance of *TP53* mutations depends on the specific type of mutation. The most frequent mutations are “missense” mutations, primarily located in exons 4-8, which encode the DNA-binding domain (DBD) of the protein. These mutations are more strongly associated with poor clinical outcomes because they directly impair p53’s ability to bind DNA and suppress tumor development. In contrast, mutations outside the DNA-binding domain have a less pronounced effect on patient survival.

Patients with *TP53* mutations in exons 5-8 face twice the risk of death following standard chemotherapy compared to those with *wild-type TP53* [24]. These mutations may be accompanied by the loss of heterozygosity (LOH) on the second allele, further exacerbating the functional loss of *TP53* and accelerating cancer progression.

The deletion of chromosome 17p, resulting in the loss of the *TP53* gene, has been less thoroughly studied in DLBCL. However, Xu-Monette et al. reported that around 12% of DLBCL cases exhibit this deletion, with a slightly higher incidence in tumors also harboring *TP53* mutations (17%) [23]. In these cases, the 17p13.1 deletion, linked to a higher proliferative index, indicating more aggressive tumor behavior.

Similarly, recent research on high-grade lymphomas has shown that the combined effect of *TP53* mutations and 17p deletion leads to full inactivation of the gene, contributing to extensive genomic instability. This enables cancer cells to escape immune surveillance and accelerates the clonal evolution of malignant B cells. As a result, the progressive accumulation of genetic abnormalities, such as aneuploidy, chromosomal rearrangements, and gene amplifications, drives the advancement of these lymphomas.

Furthermore, evidence suggests that in single hit lymphomas (SH), *TP53* alterations can act as a "second hit," predicting a poor prognosis comparable to that of patients with double-hit MYC/BCL2 lymphomas [13].

Heterogeneity in large B-cell lymphomas

To advance our understanding of large B-cell lymphomas, it is essential to address the critical topic of tumor heterogeneity. It's a crucial aspect receiving increasing attention in oncology, supported by a growing number of studies showing that heterogeneity can manifest to varying degrees, not only between different regions of the same tumor sample but also between the primary tumor and metastatic lesions in the same patient. Several factors contribute to this phenomenon, including genetic instability, epigenetic modifications, the influence of the tumor microenvironment, and stochastic variations induced by cancer therapies. In fact, all these factors can promote clonal evolution from a common progenitor clone, leading to the emergence of increasingly aggressive and treatment-resistant subclonal populations. The first published studies on inter- and intra-tumoral genetic heterogeneity were performed in the context of solid tumors but this phenomenon has been observed in a wide range of tumors, including lymphoproliferative neoplasms, and has been shown to influence clinical outcomes, disease progression and responses to treatment [25].

In fact, cytogenetic and molecular data indicate that in many mature B-cell neoplasms, the neoplastic process has prognosis correlating to the increasing complexity of the karyotype and the number of genetic alterations [26].

Literature data has established that a primary genetic alteration, often present early in tumor stem cells, plays a pivotal role in initiating lymphomagenesis. However, secondary and tertiary genetic anomalies are essential for neoplastic progression and the transformation of indolent lymphoid neoplasms into more aggressive forms. Notably, even during the early stages of the disease, secondary genetic anomalies may be present within a minor subclonal population. These subclones have the potential to expand and become dominant during disease progression or relapse.

Progression from low-grade to high-grade lymphoma is an example of intra-tumoral heterogeneity in lymphoid neoplasms. In fact, in the context of a low-grade lymphoma a subclonal population with secondary genetic anomalies, such as *MYC* rearrangements, can emerge and expand, driving the progression into more aggressive disease and profoundly influencing the clinical history of patients.

Another example of intratumoral heterogeneity is the synchronous coexistence of multiple synchronous sites, where some anatomical locations exhibit low-grade lymphoma while others show high-grade features. Less frequently, two distinct lymphomas can be found synchronously within the same organ. This rare condition is

called **composite lymphoma**, meaning a form of lymphoma in which different types of lymphoma cells occur at the same time. The different lymphoma cells, combinations of two non-Hodgkin lymphomas or a combination of a non-Hodgkin lymphoma and a Hodgkin's lymphoma, may form in the same tissue or organ or in many different tissues or organs [27].

As instance, in figure 5, a case in which, in the context of the same histological specimen, two different types of low-grade B lymphoma (CLL and FL) coexisting with high-grade transformation can be observed [28].

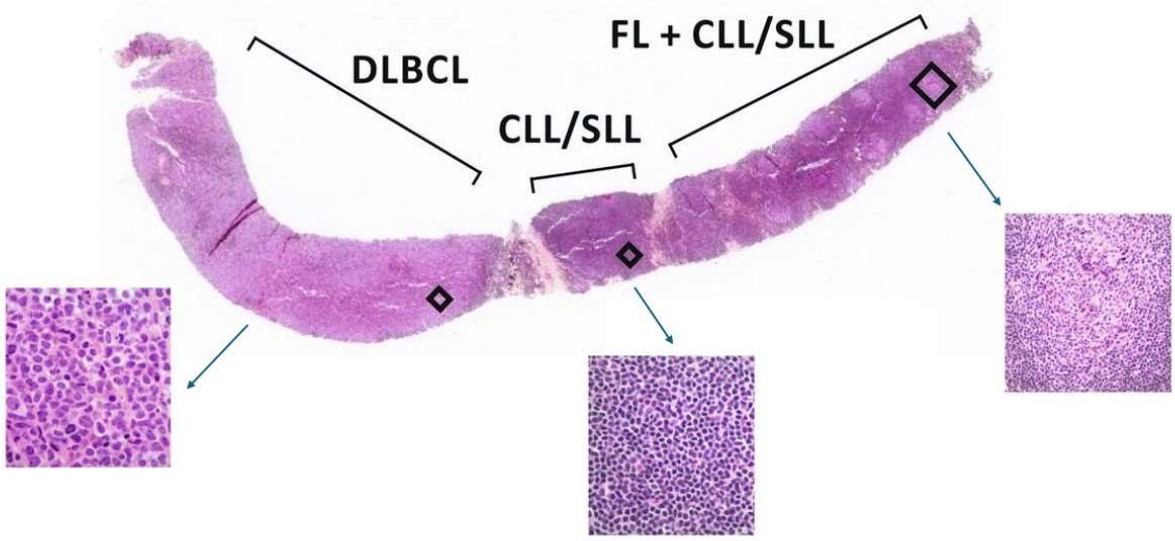


Fig. 5. Adapted from Moore, A.M., Moshkin, O., Swain, G.J. *et al*. High-grade B-cell lymphoma with *MYC* and *BCL2* rearrangements arising in composite lymphoma. *Diagn Pathol* **13**, 34 (2018). https://doi.org/10.1186/s13000-018-0714-z

Focusing on DLBCL, it is characterized by significant heterogeneity from both biological-molecular and clinical perspectives, particularly regarding treatment responses and survival rates.

As previously explained, the pioneering classification proposed in the early 2000s by Alizadeh and colleagues, based on GEP, was one of the first findings that provided a biological substrate for the heterogeneity of DLBCL, which until that moment had been evident only from a clinical perspective.

In clinical practice, usually, COO determination in DLBCL is based on immunohistochemistry algorithm, elaborating to surrogate GEP.

The most widely used of these algorithms is the one proposed by Hans in 2004 [29]. It relies on the evaluation of the expression of **CD10**, **BCL6**, and **MUM1**. According to this algorithm, the **GCB** subtype is characterized by either positive expression of **CD10** and **BCL6** with negative expression of **MUM1**, or by negative expression of **CD10**, positive expression of **BCL6**, and negative expression of **MUM1**. In contrast, the **ABC** subtype shows positive expression of **MUM1**, regardless of **BCL6** expression, and negative expression of **CD10**. In addition to Hans' algorithm, other approaches have been proposed like the one by Choi (2009) [30] and others like the so-called “tally methods” (2010) [31]. Likewise, these additional algorithms rely on similar markers, with some variations, such as the inclusion of **BCL2** in diagnostic criteria.

Another concept underlying the heterogeneity of DLBCL is that of the so-called "double-expressor" lymphoma (DEL). DEL, defined as high grade B lymphomas with an overexpression of MYC and BCL2 proteins not related to underlying chromosomal rearrangements, in clinical practice, is routinely assessed by IHC.

It’s not a distinct entity in the current World Health Organization classification but accounts for 20% to 30% of DLBCL cases and has a distinct clinical phenotype, as demonstrated by Green et al. [32]. Indeed, in their retrospective analysis, patients affected by DEL had a median age of 71 years, whereas the median age was 62 years for patients without double-hit or double-expressor DLBCL. In addition, DEL patients were more likely to have a poor performance status, advanced-stage disease, a higher Ki-67 proliferative index, intermediate/high-risk to high-risk IPI scores, multiple extranodal sites of disease, and an inferior complete response rate to R-CHOP chemotherapy.

On the contrary, intratumoral heterogeneity in HGBCL is an area of recent interest and is currently only partially understood, also considering the difficulties in obtaining sufficiently large cohorts of patients with these characteristics.

Mutational Landscape of DLBCL

As already mentioned, the first insight into the molecular heterogeneity of DLBCL arose from the application of gene expression profiling using microarray technology.

Initial unbiased genome and exome-wide sequencing studies confirmed the biological differences between ABC and GCB subtypes of DLBCL, while also uncovering genetic similarities between GCB DLBCL and FL.

Subsequently, NGS application has highlighted that DLBCLs have a greater genomic complexity compared to other hematological neoplasms, finding alterations in more than 700 different genes. However, according to data reported, the recurrently mutated genes in more than 5% of patients and considered relevant in the pathogenesis of DLBCL are approximately 150. They represent protein-coding driver genes identified as recurrently mutated or targets of somatic copy number alterations. Many recurrently mutated genes were specific to B-cell lymphoma, suggesting a distinct physiopathology from that of epithelial malignancies. Investigation of the more frequent mutations revealed lymphoma specific oncogenic mechanisms such as the mutation of chromatin modifiers (typically in GCB DLBCL) and the B-cell receptor (BCR) pathway (typically in ABC DLBCL) [33], [34].

While most of these genes are mutated in a minority of patients, the mutations are not randomly distributed.

Indeed, it has been noted that some genetic alterations have been grouped together in the same cases. For example, *MYD88* mutations are frequently associated with *CD79B* mutations, similarly *BCL2* translocation is often associated with *CREBBP* and *EZH2* gene mutations. This clustering of mutations suggests that genetic alterations could be grouped into functional clusters, potentially representing biological subtypes of DLBCL.

Staudt and colleagues at the National Cancer Institute (NCI) conducted an integrated study involving transcriptional profiling, whole-exome sequencing, targeted mutation sequencing, and array-based copy number analysis in 574 cases of DLBCL[35]. This approach revealed distinct combinations of genetic alterations within each transcriptional subtype of DLBCL, extending beyond the two main COO subgroups.

Based on this clustering, they proposed genetic subgroups based on the most common genetic features, as is listed below.

- **MCD**: characterized by ***MYD88*** and ***CD79B*** mutations, strongly associated with ABC DLBCL.
- **EZB**: enriched for ***EZH2*** mutations and ***BCL2*** translocations, was characteristic of GCB DLBCL.
- **BN2**: enriched for ***BCL6*** structural alterations and ***NOTCH2*** mutations (mostly cases unclassifiable by COO).
- **N1:** ABC DLBCL cases with ***NOTCH1*** mutations, mutually exclusive with other ABC or ***NOTCH2*** mutations.

Notably, more than half of the patients remained unclassified (UC), highlighting the possibility of additional subtypes yet to be identified.

At the same time, Shipp and colleagues at Harvard applied an unsupervised clustering approach to mutational and copy number data obtained from whole-exome and targeted sequencing of 304 DLBCL cases [10]. They identified five clusters (C1–C5), each containing cases with the most similar genetic alterations.

Interestingly, there was substantial overlap between these groups and those identified by Staudt et al.

- **C1:** enriched for ***BCL6*** fusions and ***NOTCH2*** mutations (aligned with the **BN2** group)
- **C3**: enriched for ***BCL2*** translocations and ***CREBBP*** and ***EZH2*** mutations. (aligned with the **EZB** group).
- **C5**: enriched for ***MYD88*** and ***CD79B*** mutations (aligned with the **MCD** group).

However, two additional clusters emerged:

- **C2**: dominated by ***TP53*** mutations and widespread copy number alterations,
- **C4**: enriched for somatic hypermutation of ***SGK1*** and histone linker proteins.

Interestingly, unlike Staudt's work, only a small number of cases (C0, about 4%) showed no classifiable mutations.

A follow-up study by the NCI researchers focused on unclassified cases in their initial publication and the authors noted an enrichment for tumors with high aneuploidy levels and ***TP53*** mutations. This led to the identification of two additional subtypes:

- **A53** (characterized by aneuploidy and ***TP53*** mutations)
- **ST2** (enriched for ***SGK1*** and ***TET2*** mutations)

These new clusters correspond closely to Harvard's **C2** and **C4** clusters [36].

The genetic classification system developed by the NCI became known as **LymphGen** and was released as a publicly available tool designed to classify individual cases of DLBCL based on their genetic profiles. Figure 6 shows an image showing the comparison between the classification developed by the NCI and that of Harvard.

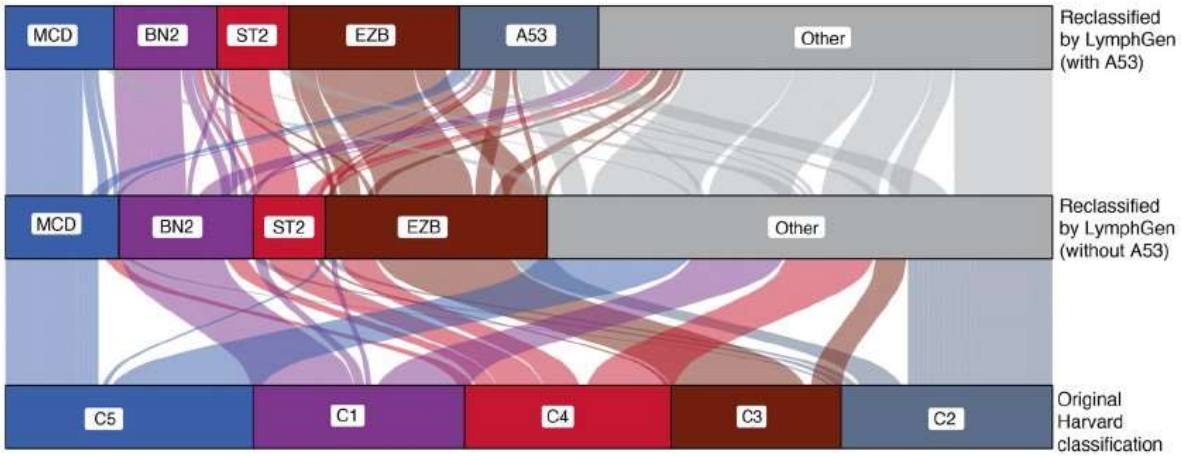


Fig. 6 comparison of the genetics and classification strategies of the NCI (LymphGen) and Harvard studies [37].

The Haematological Malignancy Research Network (HMRN) applied targeted sequencing to 928 DLBCL cases and interestingly, although this study had notable differences from the NCI and Harvard studies (sequencing strategy, types of genetic data seen by the clustering algorithms, and statistical approach), genetic subtypes emerged that could be mapped almost precisely into the NCI and Harvard categories.

These subtypes were named according to the most enriched genetic feature.

For example, there is a group called *MYD88* which corresponds to the MCD/Cluster C5, or a *BCL2* group corresponding to EZB/C3. Overall, 27% of cases remained UC.

Image 7 shows an alluvial plot showing the comparison of the modified HMRN and LymphGen classifications for individual cases from the HMRN study. It can be observed that cases classified by LymphGen are predominantly assigned to their equivalent HMRN equivalent group

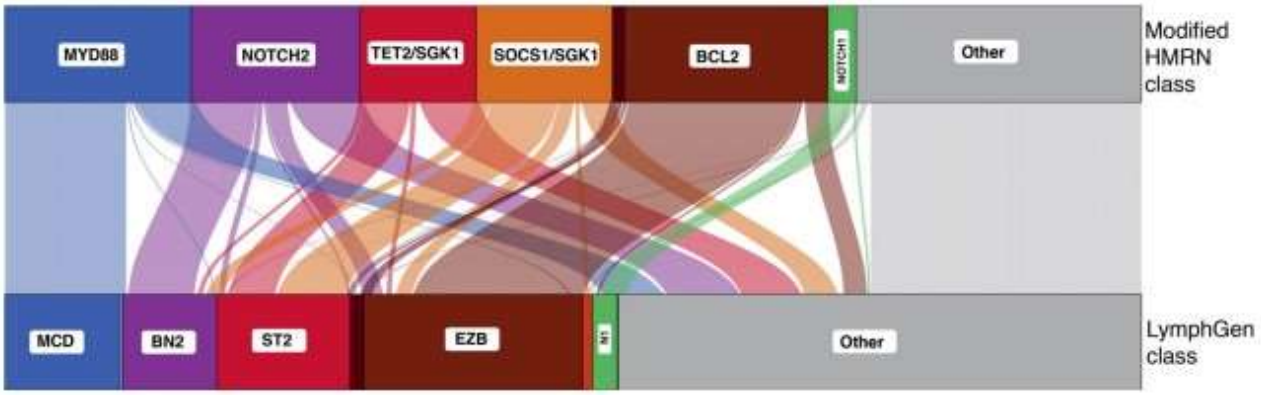


Fig. 7 comparison of the modified HMRN and LymphGen classifications for individual cases from the HMRN study [37].

It is therefore encouraging to note that, despite differences in sequencing and computational methods, there is a high degree of convergence among studies regarding the genetic classification of DLBCL [37].

In conclusion, it seems that genetic classification offers a framework to organize the genetic heterogeneity of DLBCL into subgroups with shared biological mechanisms, which may, in turn, exhibit similar responses to targeted therapies.

Risk stratification in DLBCL/HGBCL

In conclusion, emerging data strongly suggest that DLBCL/HGBCL is not a single disease entity but a spectrum of large B-cell neoplasms potentially comprising distinct morphological variants, molecular subtypes and nosological entities, with different prognosis and response to standard R-CHOP therapy.

With the routine introduction of techniques like FISH, it became possible to identify high-risk cases of large B-cell lymphoma and direct them toward more aggressive therapies than standard R-CHOP. However, the focus has now shifted to the routine introduction of newer technologies like GEP and NGS, having the potential to identify high-risk patients who might not be detected through FISH analysis of MYC/BCL2 rearrangements alone, enabling even more precise and personalized treatment strategies.

Focusing exclusively on *MYC*, *BCL2*, and *BCL6* rearrangements for risk stratification might exclude *MYC*-negative DLBCL/HGBCL patients who, through techniques like GEP or NGS, could show high-risk molecular profiles. Recent studies, including Frosch et al's work, emphasize the need for additional molecular analyses [38].

In particular, three major areas of analysis seem to contribute to a better molecularly defined risk stratification:

- Identifying *TP53* gene anomalies
- Characterizing the *MYC* partner gene
- Combining gene sequencing with gene expression techniques

Particularly, in patients with aggressive B lymphoma and *MYC* rearrangements, *TP53* gene alterations (mutations, deletions, or loss of expression) are associated with a poor prognosis and potential resistance to standard treatments [39]. Therefore, the combined analysis of mutational status, presence of deletions and protein expression of *TP53* can improve risk stratification.

Finally, in light of the latest studies, some of which have been presented in the previous sections, it is becoming increasingly clear that routine pathological diagnostics may no longer suffice to guide first-line treatment selection for patients. In parallel, there is growing evidence that the scientific community is on the brink of having more robust molecular assays available for clinical use.

If validated in clinical trials, genomic sequencing and GEP could ultimately become standard practice for risk stratification and treatment selection for patients with DLBCL or HGBCL. To fully realize their potential and improve outcomes for all patients, these assays must be universally accessible and provide results within clinically relevant timeframes.

Developing diagnostic and prognostic algorithms that synergistically integrate both traditional and novel techniques is essential to ensure optimal patient outcomes.

Aims

Since the biological and clinical diversity observed in high-grade lymphomas is underpinned by genetic and molecular variability, which deserves further investigation, in the present work we aim to identify key biological markers and molecular alterations that distinguish patient subgroups with different prognoses could facilitate the selection of more targeted therapeutic approaches.

Diagnostic tools such as immunohistochemistry and FISH, combined with advanced molecular biology techniques like genomic sequencing and gene expression profiling enables a more precise evaluation of tumor aggressiveness. Ongoing studies aim to determine the most effective combination of these methods, based on specific work algorithms, to improve risk stratification in high-grade lymphoma patients.

This study aims at refining the concept of molecular risk stratification in the specific subgroup of high-grade B-cell lymphoma patients with MYC abnormalities, encompassing not only the classic translocations t(8;14)(q24;q32), t(2;8)(p12;q24), t(8;22)(q24;q11), but also deletions, duplications, and increased copy numbers of the gene.

Materials and Methods

Patients and Tissue Samples

The study was conducted on patients with a histological diagnosis of DLBCL/HGBCL, spanning from January 1, 2015, to June 30, 2021, across various institutions throughout the Piedmont region: **Candiolo Institute - IRCCS**, Candiolo (TO), **Azienda Ospedaliera S.Croce e Carle**, Cuneo (CN), **Azienda Ospedaliera S. Luigi**, Orbassano (TO), **A.O.U. Città della Salute e della Scienza**, Turin (TO).

The inclusion criteria required DLBCL/HGBCL with the presence of *MYC* gene rearrangement or numerical abnormalities, identified using the FISH technique during the diagnostic process, and that the patients had been treated with curative intent using Rituximab-CHOP-like regimens (R-CHOP, R-COMP, DA-EPOCH-R, R-CODOX-M/R-IVAC) and other intensive treatments, including R-miniCHOP.

The major exclusion criteria included: primary mediastinal B lymphoma, Burkitt lymphoma, unclassifiable lymphoma with intermediate characteristics between DLBCL and classical Hodgkin lymphoma (so called “Grey-Zone Lymphoma”), primary effusion lymphoma, primary cutaneous DLBCL, and "leg-type” lymphoma. OS was measured from the date of lymphoma diagnosis to the date of the last follow-up visit or the date of death related to the disease. In total, 42 patients were analyzed, and clinical data were reported, including age at disease onset, sex, histological diagnosis, primary site, and follow-up data.

The mean age of the patients was 65 years, with a predominance of affected male (24 out of 42 patients, 57%) (Table 1). The additional cytogenetic and molecular analysis were carried out at Candiolo Institute – IRCCS.

All the enrolled patients signed an informed consent, approved by the ethical committee of the involved centers.

Patients ID	Diagnosis	Tumor Site	Tissue Specimens	Age	Sex
DLBCL-INN-09-01	DLBCL	Stomach	Biopsy	80	F
DLBCL-INN-09-02	DLBCL	Right Colon	Surgical specimen	71	M
DLBCL-INN-09-03	DLBCL	Left axillary lymph node	Biopsy	74	M
DLBCL-INN-09-04	DLBCL	Left inguinal lymph node	Biopsy	54	F
DLBCL-INN-09-05	DLBCL	Latero-cervical	Biopsy	57	F

		lymph node			
DLBCL-INN-09-06	DHL	kidney	Surgical specimen	71	F
DLBCL-INN-09-07	THL	Bone Marrow	Biopsy	53	M
DLBCL-INN-09-08	DLBCL	Bone Marrow	Biopsy	67	F
DLBCL-INN-09-09	DHL	supraclavicular lymph node	Biopsy	70	F
DLBCL-INN-09-10	DLBCL	Breast	Biopsy	39	F
DLBCL-INN-04_01	DHL	Paravertebral tissue	Biopsy	73	M
DLBCL-INN-04_02	HGBCL	Left inguinal lymph node	Biopsy	85	M
DLBCL-INN-04_03	HGBCL	Abdominal retroperitoneal tissue	Biopsy	80	M
DLBCL-INN-04_04	DHL	Lymph node	Biopsy	72	F
DLBCL-INN-04_06	DLBCL	Left parietal pleura	Biopsy	69	F
DLBCL-INN-04_07	DLBCL	Bladder	Biopsy	63	M
DLBCL-INN-04_08	DHL	Left supraclavicular lymph node	Biopsy	63	M
DLBCL-INN-04_09	DLBCL	Supramesocolic lymph node	Biopsy	70	M
DLBCL-INN-04_010	DHL	Right latero-cervical lymph node	Biopsy	61	M
DLBCL-INN-04_012	DHL	Inguinal lymph node	Biopsy	44	F
DLBCL-INN-04_013	DHL	Lymph node	Biopsy	57	M
DLBCL-INN-01-01	DHL	Lymph node	Biopsy	58	F
DLBCL-INN-01-03	THL	Mediastinal mass	Biopsy	65	M
DLBCL-INN-01-04	HGBCL	Jaw	Surgical specimen	41	F
DLBCL-INN-01-05	HGBCL	Lymph node	Biopsy	72	M
DLBCL-INN-01-06	DLBCL	Breast	Biopsy	55	F
DLBCL-INN-01-07	DLBCL	Bone Marrow	Biopsy	61	F
DLBCL-INN-01-08	DLBCL	Lymph node	Biopsy	72	M
DLBCL-INN-01-09	DLBCL	Bone Marrow	Biopsy	42	M
DLBCL-INN-01-10	DLBCL with MYC and BCL6 rearrangements	Lymph node	Biopsy	73	F
DLBCL-INN-01-12	DLBCL	Ileo-colon	Biopsy	63	M
DLBCL-INN-01-13	DHL	Lymph node	Biopsy	68	M
DLBCL-INN-01-14	DLBCL	Lymph node	Biopsy	54	F

DLBCL-INN-10-04	DLBCL	Stomach	Biopsy	85	M
DLBCL-INN-10-05	DLBCL	Stomach	Biopsy	64	M
DLBCL-INN-10-06	DLBCL	Colon	Surgical specimen	69	M
DLBCL-INN-10-07	DLBCL	Bowel	Biopsy	16	M
DLBCL-INN-10-09	DHL	Stomach	Biopsy	63	F
DLBCL-INN-10-10	THL	Ureter	Biopsy	71	F
DLBCL-INN-10-11	THL	Stomach	Biopsy	77	M
DLBCL-INN-10-12	DLBCL with MYC and BCL6 rearrangements	Pleural fluid	Cytological specimen	79	M
DLBCL-INN-10-14	DHL	Lung	Biopsy	82	M

Table 1. Clinico-pathological characteristics of cases enrolled in the study. DLBCL, Diffuse Lymphoma a Large B Cells; HGBCL, High-grade B-Cell Lymphoma; DH, Double Hit; TH, Triple Hit.

Immunohistochemical analysis

The COO, as determined by Hans algorithm [9], and the expression of the proliferation marker Ki67 were assessed through immunohistochemical analysis, which is routinely performed for diagnostic purpose (Fig. 8). As reported in introduction, the Hans algorithm relies on the evaluation of three markers to differentiate the GCB subtype from the non-GCB subtype. It is the most popular algorithm, but concordance with gene expression is only 72–86% [40]. Using the Hans algorithm, CD10, BCL6, and MUM1/ IRF4 are each considered positive if ≥ 30% of the tumor cells stain positive.

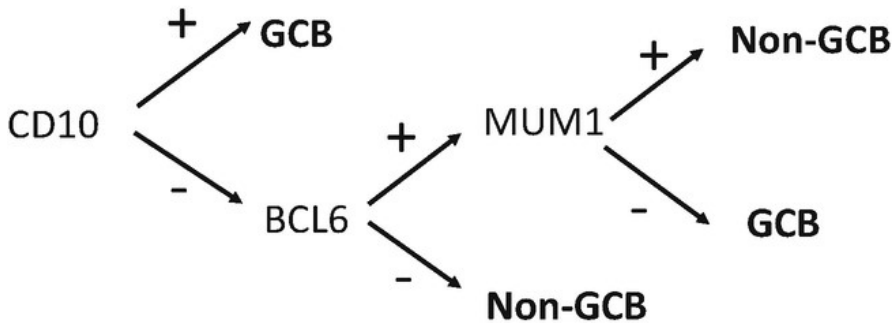


Fig. 8 Hans algorithm

Additional immunohistochemical evaluations were performed to assess key markers, including Bcl2, MYC, and p53. However, in some samples, not all markers were analyzed due to limitations in the available tissue material.

The analyses were conducted on unstained 3 µm thick histological sections from Formalin-Fixed, Paraffin-Embedded (FFPE) tissue samples, obtained from the same block used for FISH and NGS.

Fluorescence In Situ Hybridization (FISH)

The structural and numerical anomalies of the *MYC*, *IGH*, *BCL2*, *BCL6*, and *TP53* genes were analyzed using FISH performed on 4 µm thick sections of FFPE tissue, following the protocols recommended by the manufacturers. Depending on the expected type of rearrangement, various types of probes were utilized:

- Dual color break-apart probes specific for the *MYC*, *BCL2*, and *BCL6* genes (Zytovision GmbH, Germany), which are the preferred choice when multiple "partner" genes are involved.
- Dual color dual fusion probes specific for IGH-MYC rearrangements (Zytovision GmbH, Germany).
- Dual-color locus-specific probes for TP53/CEN17 (Zytovision GmbH, Germany) to assess *TP53* deletions and numerical abnormalities of chromosome 17.

The *SPEC MYC Dual Color Break Apart* probe (Zytovision GmbH, Germany) was selected due to its dimensions, considering the critical role the *MYC* gene probe could play in the sensitivity of the methodology. In fact, this probe is characterized by large dimensions (totaling 2.5 Mb) and consists of a centromeric portion of approximately 475 Kb located 385 Kb from the MYC gene and a telomeric portion of about 560 Kb situated 1.6 Mb from the gene. This design increases the probability of detecting MYC-nonIGH rearrangements, like findings reported by Chong and colleagues using similar sized probes (Fig. 9) [41].

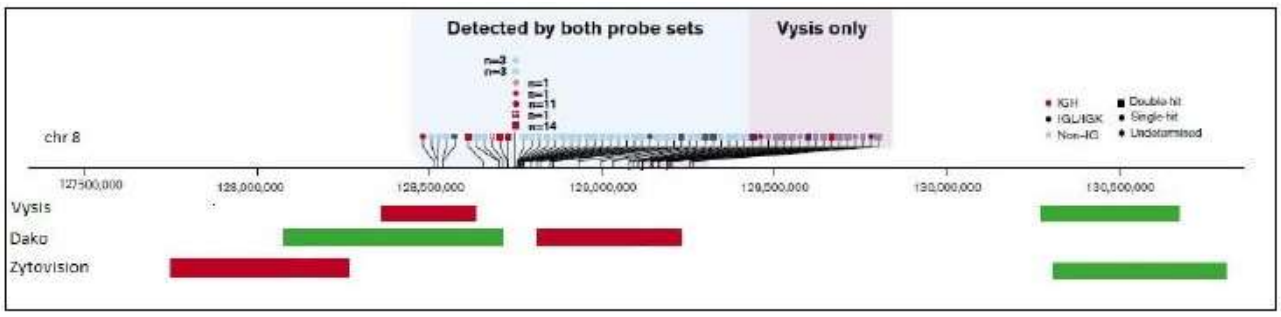


Fig. 9. Comparison between choose probes and other commercially available probes, adapted from Chong *et al*.

Regarding the processing, following deparaffinization and rehydration, the sections underwent pre-treatment and enzymatic tissue digestion with pepsin (Histology FISH Accessory Kit - Agilent, United States). This step was performed to enhance the porosity of cell membranes and "unmask" the nuclei by releasing them from the extracellular matrix and cytoplasmic components. The subsequent hybridization with the probe of interest was conducted according to the thermal profile specified by the supplier (denaturation for 10 minutes at 75°C and overnight hybridization at 37°C with controlled humidity). Next, stringency washes were performed, and counterstaining of the DNA in the nuclei was carried out using the fluorescent dye 4',6-diamidino-2-phenylindole (DAPI) (DAPI/DuraTect-Solution, Zytovision GmbH, Germany).

The analysis was performed by capturing images of the regions of interest within the lesion, pre-selected by the pathologist, at a magnification of 40X using an automatic scanning system and Metafer analysis software (MetaSystems srl, Milan, Italy), connected to a fluorescence microscope (Axio Imager epifluorescence microscope - Carl Zeiss, Oberkochen, Germany). The cut-off value used was calculated based on internal laboratory validations; for each probe employed, histological sections from five different non-pathological lymph nodes were analyzed as normal controls, selecting a cut-off value of 3 standard deviations (SD) above the mean, in accordance with European recommendations for FISH investigations on tissue samples [42].

DNA and RNA Extraction

Nucleic acids, including both DNA and RNA, were purified from 7 mm thick sections using the QIAGEN FFPE DNA kit (Qiagen, Venlo, Netherlands) and the High Pure FFPE RNA Isolation Kit (Roche, Basel, Switzerland) for DNA and RNA extraction, respectively, following the manufacturer’s protocol.

Nucleic acids quantification was performed using the Qubit 3.0 system (Thermo Fisher Scientific, Massachusetts, USA), a fluorometer measuring the fluorescence emitted by fluorimetric dyes that intercalate with the target molecules (DNA, RNA, or proteins).

RNA quality was assessed using quantitative PCR (qPCR) through the Archer Pre-Seq RNA Quality Control (QC) qPCR Assay (ArcherDX, Boulder, CO, USA), with a threshold Cq value set at <31, indicating suitable quality for downstream applications.
This process ensures that high-quality nucleic acids are obtained for further analyses, such as sequencing or expression studies, which are crucial for accurate diagnostic and research outcomes.

GEP Study to determine the COO

Samples were analyzed with the Lymph2CX assay on a NanoString (NanoString Technologies, Seattle, Washington, USA) instrument according to the manufacturer's instructions. 40 out of 42 cases could be analyzed. In two cases, there were not enough residual RNA available for analysis.
The Lymphoma Subtyping gene expression signature includes 20 genes within the NanoString Lymphoma Subtyping Test (LST) signature, 15 target genes and 5 reference genes
In the context of the LST, the quality of RNA extracted from samples is crucial to ensure the reliability of the results. To assess RNA quality, the algorithm employs the geometric mean of five housekeeping genes (HK), which are characterized by stable and constant expression across all cells.
These housekeeping genes serve as reference standards because their expression remains unchanged in response to variations in external conditions. The geometric mean (HK geomean) of the expression levels of these genes provides an indicator of the integrity and quality of the RNA in the samples. If this geometric mean exceeds the predefined clinical quality threshold of 128, the RNA is of adequate quality for analysis; otherwise, the sample may be deemed unsuitable for accurate analysis.
An HK geomean value below 64 was deemed as insufficient RNA quality to provide a subtyping result. A value between 64 and 128 is borderline quality since it meets previously published thresholds for RNA quality within clinical research studies. 2 samples out of 42 did not have enough RNA to be submitted for the analysis. All the 40 (40/40, 100%) cases passed the QC threshold. Each sample surpassing the QC threshold was reported as one of the molecular subtypes, ABC, GCB, or UC within an equivocal zone. Data were analyzed by Nanostring.

DNA sequencing for mutational analysis

High-depth sequencing was performed on 42 FFPE samples, with an input of 80 ng DNA, using the TruSight Oncology (TSO) 500 panel (Illumina, San Diego, USA) following the manufacturer's protocol. The designated panel includes 523 genes, covering a total genomic region of 1.94 Mb, enabling the assessment of somatic mutations (SNV, Indel), copy number variations (CNVs) in 59 genes, microsatellite instability (MSI) status across 120 loci, and tumor mutational burden (TMB).

The selection of this targeted sequencing panel was based on its proven performance and robustness when applied to DNA extracted from FFPE samples. Library preparation was followed by sequencing on a NovaSeq 6000 instrument (Illumina, San Diego, California, USA) to achieve a minimum read depth of 500×. A validated in-house variant calling pipeline was used.

The LymphGen 2.0 probabilistic classification tool was adopted to categorize cases into the newly defined genetic subtypes, according to Wright et al. [36]. LymphGen is an algorithm that calculates the probability of a DLBCL belonging to one of six genetic subtypes and assigns it accordingly. It is designed to handle various input data, such as mutations, copy number variations, and BCL2/BCL6 rearrangements. This flexibility ensures LymphGen remains effective even when some data are missing.

Statistical analysis

The statistical analyses were performed using R software. Overall survival was evaluated using the Kaplan-Meier method and analysed with the Log-Rank (Mantel-Cox) test; the estimate was limited to the longest survival time if censored. A P-value of <0.005 was considered statistically significant.

Results

Cohort composition and clinical findings

A total of 42 patients participated in the study (Table 2), all of whom received first-line treatment with R-CHOP-like regimens. The median follow-up period was 36 months, allowing for an in-depth observation of treatment outcomes and potential long-term side effects. The median age of the participants was 65 years.

Regarding risk stratification, 15 out of 42 patients (36%) were classified in the low/intermediate IPI risk category, while 26 out of 42 (62%) were classified as intermediate-high/high IPI risk. Follow-up data were unavailable for 1 patient of the cohort.

Main clinical data are summarized in Table 2.

ID	Età	Gender	B symptoms	ECOG-PS	Stage	IPI (0-5)	IPIaa (0-3)	CNS-IPI (0-7)	First Line Therapy
DL-09-01	80,0	F	nd	3	IV	4	nd	4	Other
DL-09-02	71,0	M	N	1	IV	2	nd	2	R-CHOP21
DL-09-03	74,0	M	Y	3	IIISB	4	nd	4	Other
DL-09-04	54,0	F	N	0	IIIIA	2	1/2	1	R-CHOP21
DL-09-05	57,0	F	N	nd	nd	2	2	2	R-CHOP21
DL-09-06	72,0	F	Y	1	IV	4	nd	5	Other
DL-09-07	53,0	M	Y	0	IV	2	2	2	Magrath
DL-09-08	67,0	F	N	1	IV	2	nd	2	R-CHOP21
DL-09-09	70,0	F	N	2	IV	5	nd	5	Other
DL-09-10	39,0	F	N	0	I (breast)	1	1	1	R-CHOP21
DL-04-01	73	M	Y	2	IV	5	nd	5	R-DA-EPOCH
DL-04-02	85	M	N	0	IV	3	nd	3	Vincristina+R-miniCHOP

DL-04-03	80	M	Y	2	IV	5	nd	6	R-mini CHOP
DL-04-04	72	F	Y	2	IV	5	nd	5	APO
DL-04-06	69	F	N	2	IV	4	nd	4	R-DAOX+Lenalidomide
DL-04-07	63	M	N	0	IV	3	nd	3	R-CHOP+R-DA-EPOCH+RT
DL-04-08	63	M	N	0	IV	3	nd	3	R-CHOP+GMALL+RT
DL-04-09	70	M	N	1	IV	3	nd	3	R-CHOP
DL-04-010	61	M	Y	0	II	2	nd	2	R-CHOP+R-DA-EPOCH+RT
DL-04-012	44	F	N	0	IV	1	1	1	R-CHOP+RT
DL-04-013	57	M	N	2	IV	4	3	4	R-CHOP+GMALL
DL-01-01	58	F	Y	0	III	2	2	2	GMALL
DL-01-03	65	M	Y	2	IV	4	nd	4	Other
DL-01-04	41	F	N	0	IV	1	1	1	Magrath
DL-01-05	72	M	N	0	IV	2	nd	2	Other
DL-01-06	55	F	N	0	IV	3	2	4	Magrath
DL-01-07	61	F	N	0	IV	4	nd	4	R-CHOP21
DL-01-08	72	M	N	0	III	2	nd	2	R-CHOP21
DL-01-09	42,0	M	Y	0	IV	1	1	1	R-CHOP21
DL-01-10	73	F	N	0	.	2	nd	2	R-CHOP21

DL-01-12	63	0	Y	2	IV	3	nd	3	R-CHOP21
DL-01-13	68	0	Y	0	IV	4	nd	4	Magrath
DL-01-14	54	F	Y	2	IV	5	nd	5	R-CHOP21
DL-10-04	85	0	Y	1	IVB	3	1	5	R-COMP
DL-10-05	64	0	Y	1	IVB	3	2	2	R-EPOCH
DL-10-06	65	0	Y	1	IIB	3	2	3	R-COMP
DL-10-07	16	0	Y	0	IVB	3	1	2	Other
DL-10-09	64	1	Y	1	IVB	4	2	3	R-CODOX-M I-IVAC
DL-10-10	71	1	Y	1	IVA	nd	nd	nd	Other
DL-10-11	77	0	Y	1	IVB	4	2	6	R-COMP
DL-10-12	79	0	Y	1	IVB	3	2	3	R-COMP
DL-10-14	82	0	Y	1	IVA	2	2	3	R-miniCHOP

Table. 2 main clinical data of patients.

Immunohistochemical characterization

Immunohistochemical analysis was performed (as required by routine diagnostic procedures, following WHO guidelines [12]) to assess the expression of CD10, Bcl6 and MUM1/IRF4 molecules. Immunohistochemical data for all three markers were available for 40 out of 42 cases (95%), allowing the application of the Hans algorithm. For two cases, the samples were insufficient to analyze all the markers needed. Of these 40 cases, 25 were classified as GCB-type, while 15 were classified as non-GCB-type. For a subset of cases in the cohort, additional immunohistochemical markers of interest, such as Bcl2 and MYC, were also evaluated, enabling the identification of the DEL, defined as overexpression of Myc and Bcl2 proteins not related to underlying chromosomal rearrangements [43]. The expression of the Bcl2 protein was evaluated in 41 cases out of 42 and found to be positive in 36 cases (36/41; 88%) and no expression was assessed in 5 cases (5/41; 12%).

The expression of the Myc protein was evaluated in 21 cases and found to be positive in 17 cases (17/21; 81%) and negative in 4 cases (4/21 19%). Consequently, 17 cases were referable to DE lymphoma. The expression of Ki67, as an indicator of proliferative activity, was generally found to be high, with values ranging from a minimum of 40% to 100%. As is well established, high Ki67 expression is commonly associated with increased cell proliferation and can indicate aggressive behavior in various malignancies, making it a critical factor in tumor characterization and prognosis [44].

A summary of the immunohistochemical data is presented in Table 3, while in figure 10, a pie chart shows the distribution of the cases in the cohort.

ID	Ki-67 (%)	CD10	BCL-6	MUM1	COO by Hans	BCL-2	BCL-2 exp (%)	MYC IHC	MYC exp (%)
DL-09-01	>90	1	1	nd	GCB	1	nd	nd	nd
DL-09-02	90	1	1	nd	GCB	0	0	nd	nd
DL-09-03	60	1	1	nd	GCB	0	0	nd	nd
DL-09-04	40-70	0	1	nd	nd	1	nd	nd	nd
DL-09-05	95	0	1	nd	nd	1	nd	nd	nd
DL-09-06	>90	1	0	nd	GCB	nd	nd	nd	nd
DL-09-07	80	1	1	nd	GCB	1	nd	nd	nd
DL-09-08	40	1	nd	nd	GCB	1	nd	nd	nd
DL-09-09	90	1	1	nd	GCB	1	nd	nd	nd
DL-09-10	80	1	1	nd	GCB	1	nd	nd	nd
DL-04-01	>80	1	1	0	GCB	1	nd	nd	nd
DL-04-02	>90	0	1	1	ABC	0	0	nd	nd
DL-04-03	>80	0	1	1	ABC	1	nd	nd	nd
DL-04-04	>80	1	1	0	GCB	1	nd	nd	nd
DL-04-06	>90	1	1	0	GCB	1	nd	nd	nd
DL-04-07	nd	0	1	1	ABC	1	nd	1	40
DL-04-08	75	0	0	0	ABC	1	nd	1	50
DL-04-09	>80	0	1	0	GCB	0	0	nd	nd
DL-04-010	65-70	1	1	0	GCB	1	nd	nd	nd
DL-04-012	>90	1	1	0	GCB	1	nd	nd	nd
DL-04-013	>90	1	1	0	GCB	1	nd	nd	nd
DL-01-01	50	1	1	0	GCB	1	nd	0	30%
DL-01-03	80	1	1	1	GCB	1	nd	1	nd
DL-01-04	95	1	1	1	GCB	1	nd	1	nd
DL-01-05	50	1	1	0	GCB	1	>95	1	>90
DL-01-06	99	0	1	1	ABC	1	nd	1	70

DL-01-07	90	0	1	1	ABC	1	nd	1	50
DL-01-08	90	1	1	nd	ABC	1	nd	0	0
DL-01-09	70	1	1	0	GCB	0	nd	0	0
DL-01-10	70	0	1	1	ABC	1	nd	0	0
DL-01-12	90	0	0	1	ABC	1	nd	nd	nd
DL-01-13	60	1	1	0	GCB	1	nd	nd	nd
DL-01-14	90	1	1	1	GCB	1	nd	1	70
DL-10-04	90	0	1	1	ABC	1	nd	1	nd
DL-10-05	80	0	1	1	ABC	1	nd	1	nd
DL-10-06	80	0	1	1	ABC	1	nd	1	nd
DL-10-07	80	0	1	1	ABC	1	nd	1	nd
DL-10-09	90	0	1	1	ABC	1	nd	1	nd
DL-10-10	90	0	1	1	ABC	1	nd	1	nd
DL-10-11	90	1	1	1	GCB	1	nd	1	40
DL-10-12	90	1	1	1	GCB	1	nd	1	nd
DL-10-14	80	1	1	1	GCB	1	nd	1	nd

Table 3. GCB: Germinal Center B Cell, ABC: Activated B Cell; 1: expression, 0: negativity.

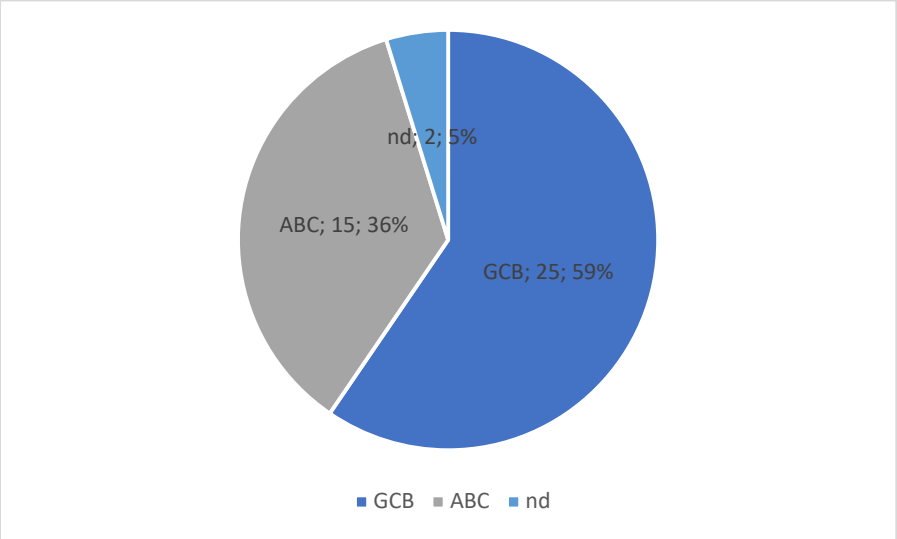


Fig. 10 Pie-chart of COO distribution according to IHC data.

Fluorescent in Situ Hybridization results

MYC, Bcl2, Bcl6

MYC rearrangement (MYC-r) was observed in thirty-four out of forty-two (34/42; 81%) patients, highlighting the predominance of structural alterations of the *MYC* gene in this population. In contrast, the increased copy number of *MYC* was detected in eight out of

forty-two (8/42; 19%) patients, indicating a notable presence of *MYC* amplification in a subset of the cohort. Among the *MYC*-r patients, thirty-one out of thirty-four (31/34; 91%) had sufficient material for further analysis, allowing a more detailed investigation of the rearrangement.

Notably, *MYC*-r involving an immunoglobulin (IG) gene was detected in eighteen out of thirty-one (18/31; 58%) of the patients, which suggests a frequent association between *MYC* rearrangements and *IG* genes, potentially indicative of a more aggressive tumor subtype. These data, in line with literature [45] underscores the importance of the *MYC*::*IG* fusion as a characteristic feature in this cohort, which is often linked with high-risk lymphomas. The remaining thirteen MYC-r cases analyzed (13/31; 42%) in the cohort involved a non-immunoglobulin (non-IG) rearrangement partner (Fig. 11). Independent of the *MYC* translocation data, *MYC* gain status was evaluable in 25 out of 42 (51%) cases in the cohort, with *MYC* gain detected in 17 of the 25 cases analyzed (17/25; 68%). In all cases, the copy number ranged from 2 to 4.

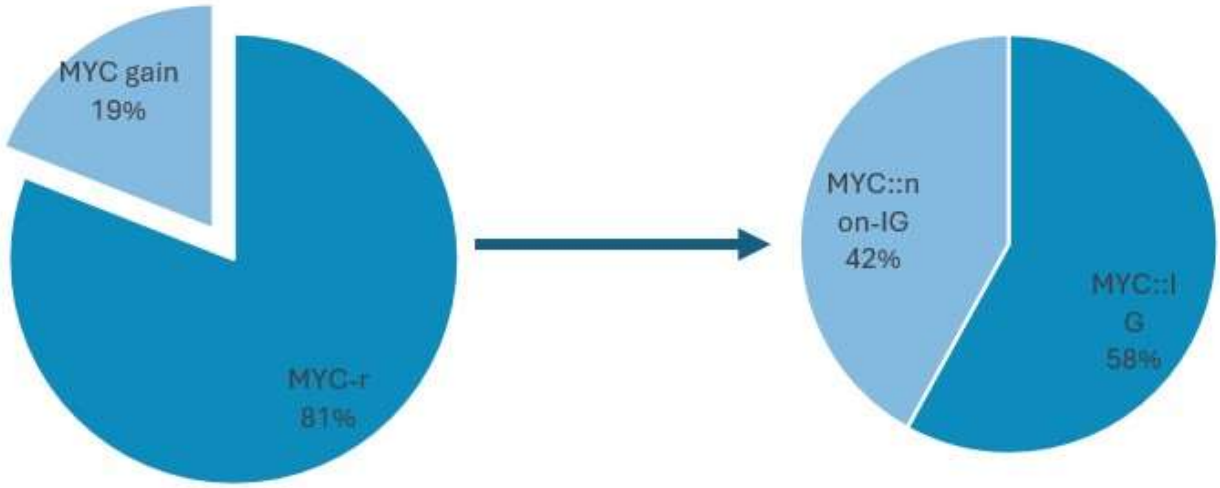


Fig. 11 Pie-chart of *MYC* anomalies distribution in the cohort.

Furthermore, *BCL2* and *BCL6* rearrangements were detected in twenty out of thirty-nine (20/39; 51%) and fourteen out of thirty-nine (14/39; 36%) patients, respectively. The co-occurrence of *MYC* and *BCL2* rearrangement was found in twelve out of thirty-one (12/31; 39%) patients, while *MYC* and *BCL6* rearrangements coexisted in six out of thirty-one (6/31; 19%) patients. Additionally, four out of thirty-one (4/31; 13%) patients exhibited rearrangements in all three genes (*MYC*, *BCL2*, and *BCL6*), reflecting a subset of patients with a more complex genetic profile that may be associated with a

particularly poor prognosis, as suggested by the previously used definition of "double-hit" or "triple-hit" lymphomas.

TP53

A *TP53* deletion was identified in seven out of forty (7/40; 18%) patients for whom this analysis was available, indicating a significant proportion of patients with a critical tumor suppressor gene deletion. The presence of *TP53* deletions is an important finding, as this alteration is often linked to more aggressive disease and a poorer OS [46]. These genetic insights offer valuable prognostic information, suggesting that the co-occurrence of *MYC*, *BCL2*, *BCL6*, and *TP53* alterations may help in identifying patients who are at higher risk and could benefit from more targeted or intensive therapeutic strategies. In three cases where no evidence of *TP53* deletion was observed, an increase in the copy number, instead, was detected.

Gene Expression Profiling results

The COO classification, a fundamental approach for categorizing aggressive B-cell lymphoma, is based on the molecular features of the tumor cells and its evaluation relies on gene expression profiling as gold standard [47]. The GCB subtype originates from germinal center B-cells, while the ABC subtype derives from post-germinal center B-cells, each with distinct biological behaviors and implications for prognosis. Some cases remain unclassified due to overlapping or ambiguous molecular features.

In our cohort of forty-two patients, the test could be performed in forty cases (40/42; 95%). In two cases (2/42; 5%), there was an insufficient quantity of residual RNA for further analysis. Of the forty cases analyzed, COO classification identified the GCB subtype in thirty-one cases (31/40; 78%), the ABC subtype in six cases (6/40; 15%), and three cases (3/40; 7%) as unclassified.

When comparing the GEP results with the IHC results obtained using the Hans algorithm, four cases could not be evaluated due to insufficient data (two due to lack of IHC data, two due to lack of GEP data). Among the thirty-eight cases where a comparison was possible, twenty-seven (27/38; 71%) showed concordance between GEP and IHC results, while eleven (11/38; 29%) were discordant. We summarized in Table 4 the comparative data of the COO, determined using Hans' algorithm from IHC data and the GEP method,

while Figure 12 provides a graphical representation through a pie chart of the rate of concordance.

ID	COO by Hans	LST Nanostring	Concordance
DL-09-01	GCB	GCB	YES
DL-09-02	GCB	GCB	YES
DL-09-03	GCB	GCB	YES
DL-09-04	nd	GCB	nd
DL-09-05	nd	ABC	nd
DL-09-06	GCB	GCB	YES
DL-09-07	GCB	GCB	YES
DL-09-08	GCB	GCB	YES
DL-09-09	GCB	GCB	YES
DL-09-10	GCB	GCB	YES
DL-04_01	GCB	GBC	YES
DL-04_02	ABC	UNCLASSIFIED	NO
DL-04-03	ABC	nd	nd
DL-04-04	GCB	GBC	YES
DL-04-06	GCB	UNCLASSIFIED	NO
DL-04-07	ABC	ABC	YES
DL-04-08	ABC	ABC	YES
DL-04-09	GCB	GBC	YES
DL-04-010	GCB	GBC	YES
DL-04-012	GCB	GBC	YES
DL-04-013	GCB	nd	nd
DL-01-01	GCB	GCB	YES
DL-01-03	GCB	GCB	YES
DL-01-04	GCB	GCB	YES
DL-01-05	GCB	GCB	YES
DL-01-06	ABC	ABC	YES
DL-01-07	ABC	ABC	YES
DL-01-08	ABC	GCB	NO
DL-01-09	GCB	GCB	YES
DL-01-10	ABC	GCB	NO
DL-01-12	ABC	GCB	NO
DL-01-13	GCB	GCB	YES
DL-01-14	GCB	GCB	YES
DL-10-04	ABC	GCB	NO
DL-10-05	ABC	UNCLASSIFIED	NO
DL-10-06	ABC	GCB	NO
DL-10-07	ABC	GCB	NO
DL-10-09	ABC	GCB	NO
DL-10-10	ABC	GCB	NO
DL-10-11	GCB	GCB	YES
DL-10-12	GCB	GCB	YES
DL-10-14	GCB	GCB	YES

Table 4; COO assessed by IHC and GEP. 0: not expressed; 1: expressed; nd: Not Determined.

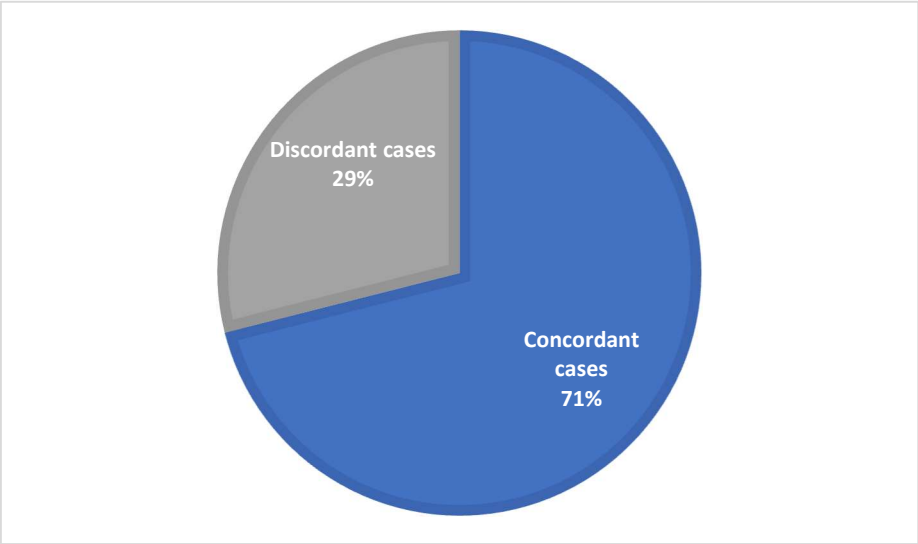


Fig. 12 Pie chart of concordance rate between IHC and GEP.

Mutational analysis using DNA sequencing techniques

In our cohort, to achieve a comprehensive mutational analysis, we selected The TruSight Oncology 500 (TSO500), taking advantage of its ability to work with FFPE samples, which allowed us to generate a detailed molecular profile for each patient.

This method enabled the identification of numerous pathogenic variants in this cohort, with the most frequently observed ones (identified at least in 15% of patients) listed below:

- *TP53* (14/42; 33%)
- *CREBBP* (13/42; 31%)
- *MYC* (13/42; 31%)
- *BCL2* (8/42; 19%)
- *TNFRSF14* (8/42; 19%)
- *CIC* (7/42; 17%)
- *EZH2* (7/42; 17%)
- *NF1* (7/42; 17%)

Among the fourteen cases with *TP53* pathogenetic alterations, eleven missense mutations (11/14; 78.6%), two splice donor mutations (2/14; 14.3%), and one frameshift mutation (1/14; 7.1%) were identified.

The high prevalence of *TP53* mutations, alongside other alterations such as those in *CREBBP*, *MYC*, and *BCL2*, underscores a complex molecular landscape among patients, emphasizing the potential influence of these varied genetic changes on disease behavior and outcomes.

Figure 13 presents an oncoprint visualizing these genetic alterations, revealing the distribution and frequency of significant mutations across the cohort. This visualization offers a comprehensive overview of the molecular characteristics observed within the study group, enhancing our understanding of patient-specific genetic profiles and potentially informing prognostic models.

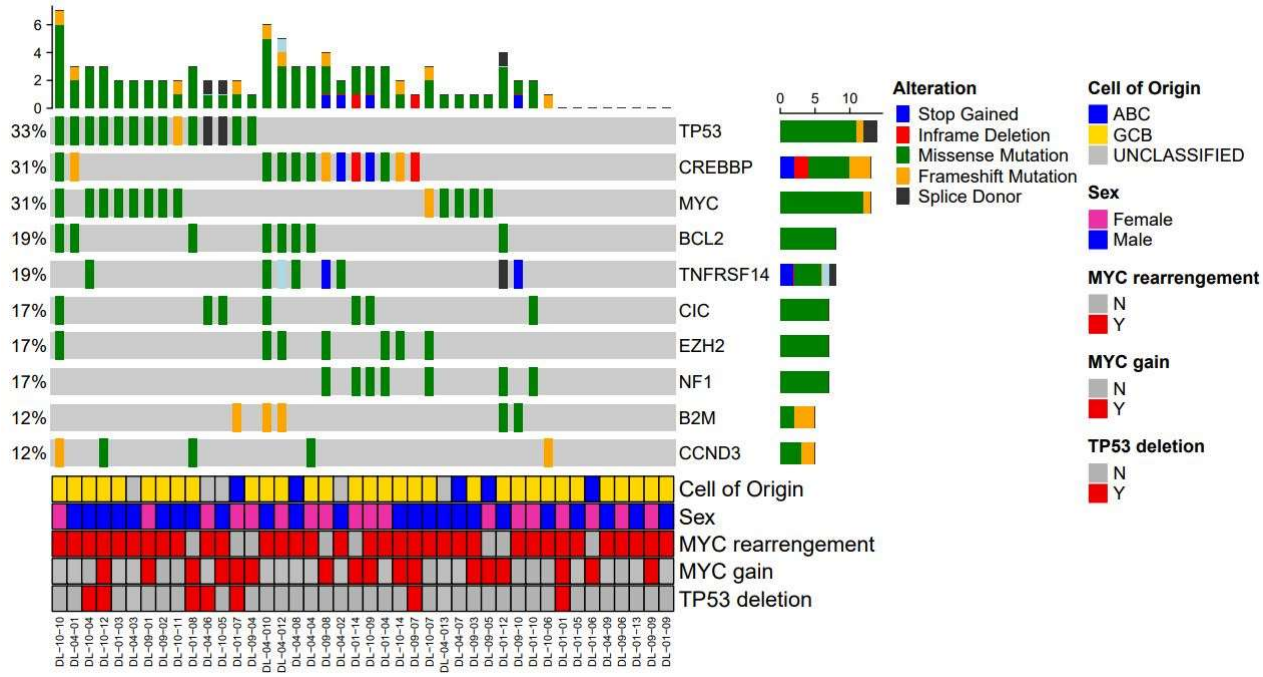


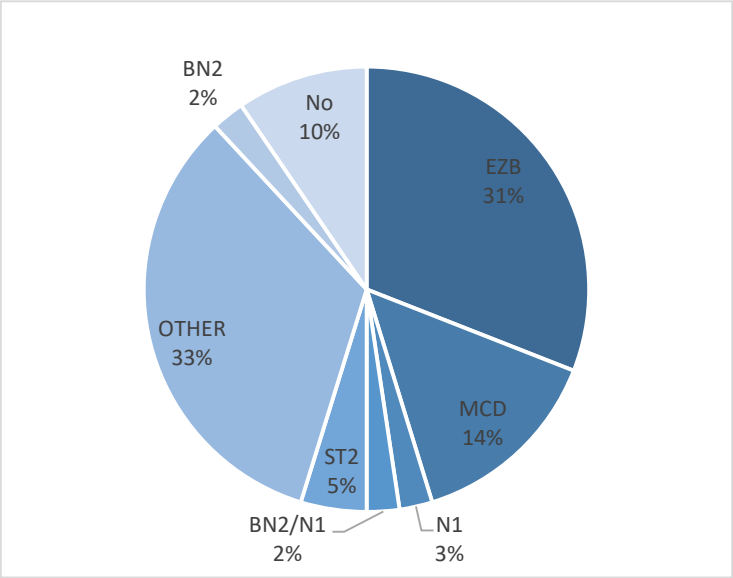
Fig. 13 Oncoprint of mutations found in association with age, proliferative index, histotype, COO, sex, the presence of rearrangement and/or increased copy number of *MYC* and the presence of *TP53* deletion.

Subsequently, molecular data were applied to the LymphGen algorithm, a predictive tool designed to assess the risk and prognosis of lymphoma patients based on their genetic profile [36].

As previously mentioned, the LymphGen algorithm determines the likelihood of a DLBCL sample belonging to any of six defined genetic subtypes and assigns the sample to one or more subtypes based on these probabilities. The algorithm was successfully applied to 38 out of 42 cases (90%). Three wild-type cases had too few variants to be assigned appropriately to a subgroup (consistently with the sequencing data). For one case, the necessary data for analysis were unavailable.

The remaining 38 cases were evaluated and distributed as follows:

- **EZB**: 13 cases, (34%).
- **MCD**: 6 cases, (16%).
- **N1**: 1 case, (3%).
- **BN2/N1**: 1 case, (3%).
- **ST2**: 2 cases, (5%).
- **OTHER**: 14 cases, (37%).
- **BN2**: 1 case, (3%).



The comparison between COO and LymphGen in this cohort was possible for 36 cases. The GCB group constitutes the majority of cases (28 /36; 77.8%). Within the GCB group, the EZB subtype is the most prevalent, comprising 10 cases (35.7%), consistent with its typical association with the germinal-centre phenotype [48]. The “Other” category also includes a significant portion of cases (8 cases, 28.6%), highlighting either classification challenges with LymphGen or molecular heterogeneity within this group. The ABC group, which is less represented with 5 out of 36 cases (13.9%), predominantly falls into the “Other” category, accounting for 3 out of 5 cases (60%). This underscores the molecular complexity of this profile. Only one case of MCD is identified, aligning with the known association between MCD and the ABC phenotype.

The unclassified cases, totalling 3 out of 36 (8.3%), still show some alignment with specific molecular subtypes such as EZB and ST2. This suggests that even in the absence of a defined COO profile, LymphGen provides valuable molecular insights. In the graphs below a graphical representation of molecular subgroup distribution depending on the different COO assigned by Nanostring (Fig. 14).

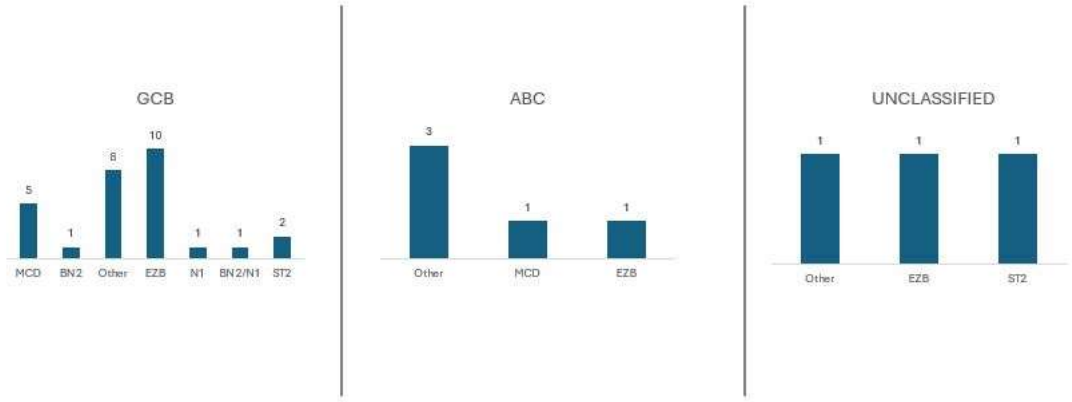


Fig. 14 Distribution of molecular signature according to COO.

Statistical correlations between molecular and clinical data

The follow-up time for patients was assessed, with the minimum follow-up being 1 month (21 days), the maximum being 111 months, and the median follow-up being 40 months. The survival analysis was conducted using the Kaplan-Meier method to estimate survival probabilities, and the survival curves were plotted to compare the differences between different groups. The log-rank test was used to assess statistical significance between the survival curves, and a p-value less than 0.05 was considered statistically significant

The 3-year OS, evaluated based on stratification from GEP data, did not identify a statistically significant difference between the GCB and ABC groups ($p = 0.4$).

As expected, patients with *MYC* rearrangements had a three-year OS rate of 56%, suggesting a moderate survival outlook overall. On the other hand, those with *MYC* gain alone showed a higher three-year OS of 75%. Even though this difference did not reach statistical significance ($p = 0.32$), this trend indicates that *MYC* gain may be associated with a slightly more favorable outcome than MYC-r alone, though further studies are necessary to confirm this observation.

Notably, in line with the existing literature, regarding MYC-r patients, the *IGH* fusions partner group presented a significantly lower three-year OS rate (33%), compared to 76% in the non-*IG* fusion partner group with statistical significance ($p=0.02$) (Fig. 15).

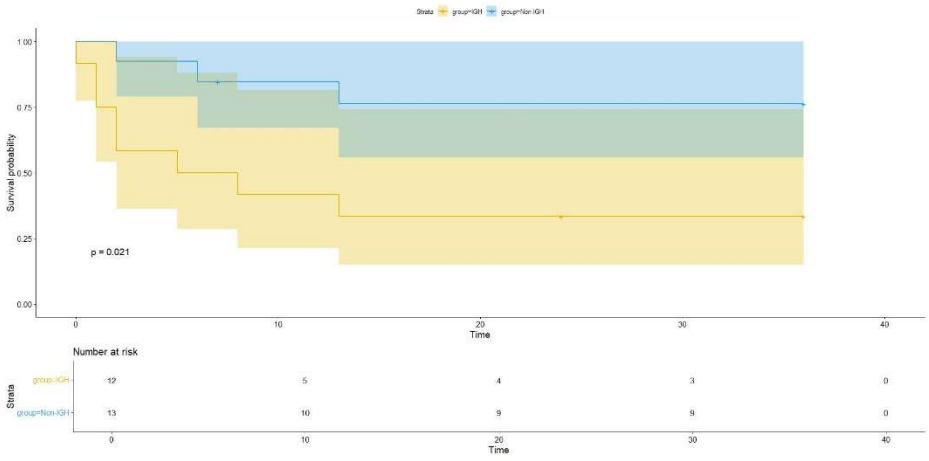


Fig.15

The presence of concomitant *BCL2* and/or *BCL6* rearrangements alongside *MYC* appeared to worsen OS outcomes further. In fact, patients with concomitant *MYC* and *BCL2* and/or *BCL6* rearrangements had a three-year OS of only 46%, compared to 75% in patients with only *MYC* anomalies (p = 0.39). This trend, though not statistically significant, confirms that concurrent *BCL2*/*BCL6* rearrangements may add a layer of complexity that impacts the overall prognosis negatively, aligning with findings in the literature linking dual-hit or triple-hit lymphomas to poor survival [7], [49].

Following these analyses, we evaluated the potential prognostic impact of cases with both a MYC-r and a *TP53* deletion.

Notably, a significant impact on OS was observed in patients with MYC-r combined with a *TP53* deletion, who had a three-year OS of just 20%, a substantial drop when compared to those without *TP53* deletion but with MYC-r (three-year OS 59%) or *MYC* gain (three-year OS 75%) (p = 0.047).

Moreover, following these preliminary analyses, we assessed whether there was a prognostic impact in cases with both a *MYC* abnormality and a *TP53* deletion, as well as in cases with a *TP53* mutation. These cases with *TP53* mutations showed high-risk disease, as demonstrated by the reduced OS (35%) compared to the other cases (73%) with a statistically significant difference (p=0.016) (Fig. 16).

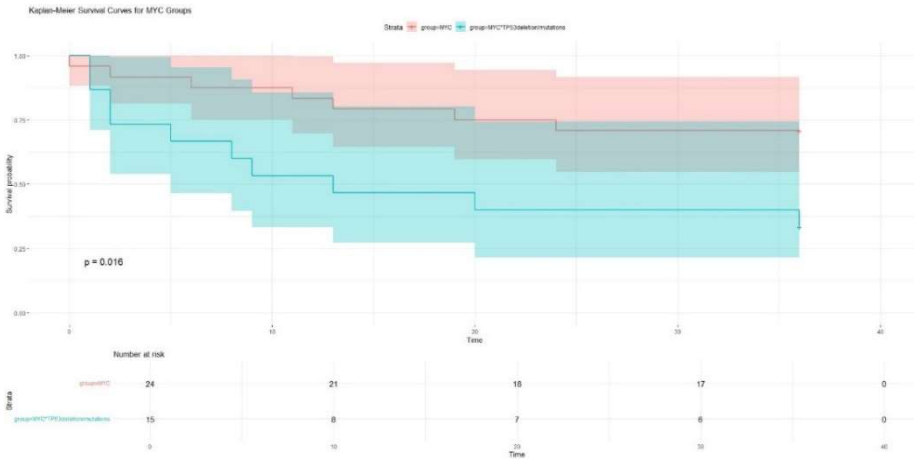


Fig. 16

Subsequently, we evaluated the presence of potential differences in OS stratifying the cohort based on the presence or absence of frequently observed mutations. Specifically, we focused on *MYC* and *CREBBP* mutations. *MYC* mutated cases showed a reduction in OS (36% instead of 68%), with statistically significant results (p= 0.021).

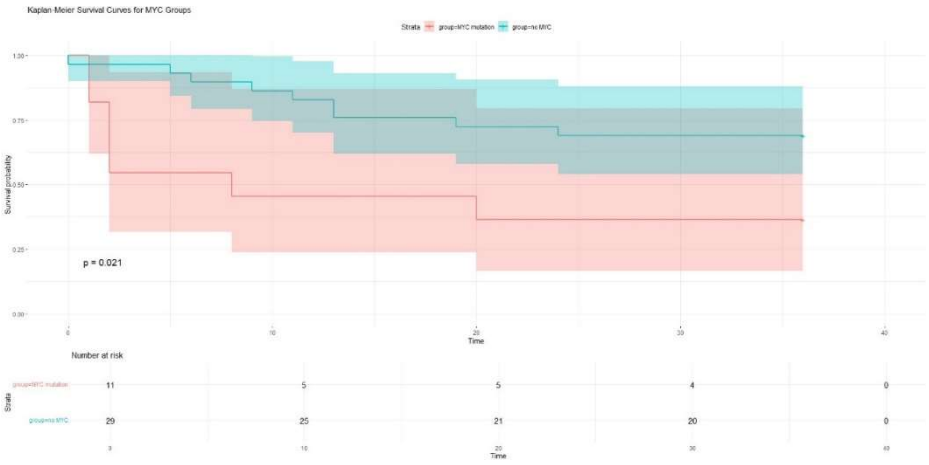


Fig. 17 *MYC* mutated vs non mutated patients

We also attempted to stratify the cohort based on *CREBBP* gene mutations. Cases with this mutation showed a higher three-year OS, although the result was not statistically significant (p=0.24).

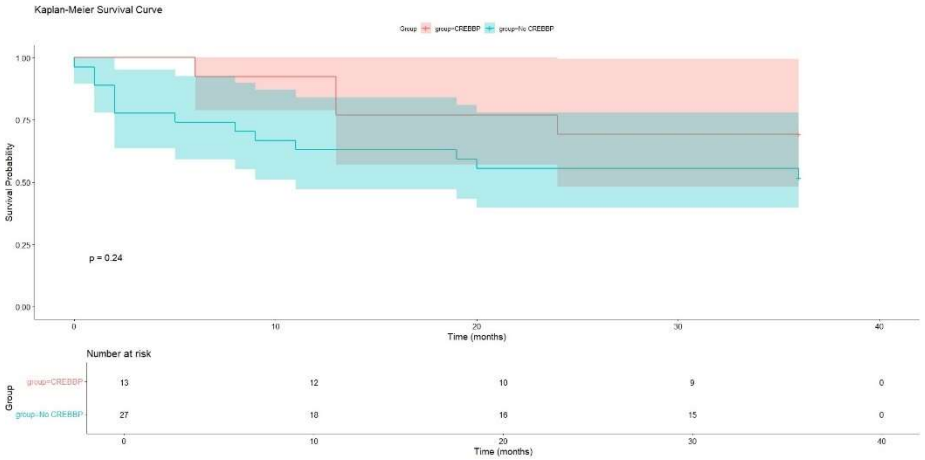


Fig. 18 CREBBP mutated vs non mutated patients.

So, the three-year OS data reveals notable differences in patient outcomes based on genetic alterations, providing insights into the prognostic impact of specific molecular mutations.

Finally, we used Kaplan-Meier analysis to stratify patients based on OS according to the signatures identified by *Lymphgen*.

Only those with sufficient numerical significance (EZB, MCD, and the 'Other' category, which includes unclassified cases) were considered.

However, none of the survival curves shown in Figure 19 below present statistically significant differences.

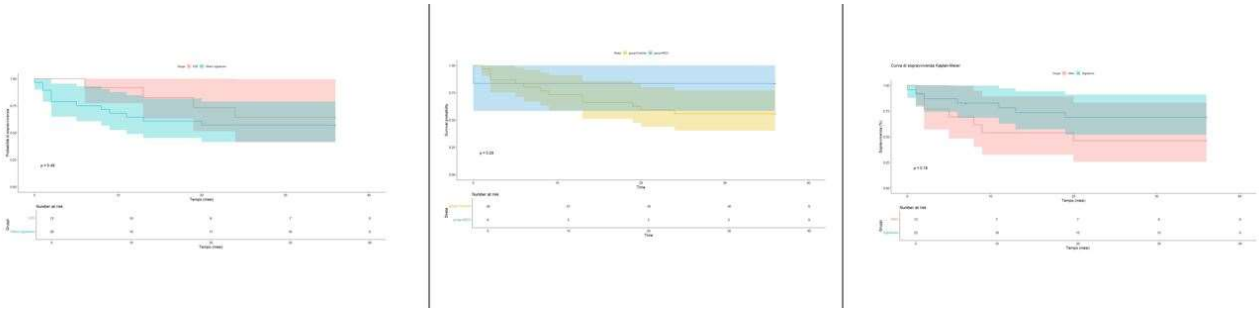


Fig.19 Kaplan Meyer referred to molecular signatures.

Discussion

The diagnostic process in DLBCL/HGBCL is primarily handled by morphological and immunohistochemical analyses.

The definition of COO, as demonstrated by numerous studies [8], is crucial for its prognostic impact. This is especially true in the ABC-DLBCL subgroup, which tends to exhibit more aggressive clinical behavior compared to GCB-DLBCL.

More recently, cytogenetic and molecular methodologies have been introduced in the diagnostic process of lymphomas, particularly DLBCL and HGBCL, to detect the rearrangements of *MYC*, *BCL2*, and *BCL6* genes. The presence of these rearrangements leads to the reclassification of HGBCL, NOS, as HGBCL with *MYC* and *BCL2* rearrangements. The role of the *MYC* gene is crucial in the clinical setting of DLBCL/HGBCL, even in cases of isolated rearrangement (SH lymphomas) or when numerical, rather than structural, anomalies are present; studies have shown that patients with isolated *MYC* rearrangement and variations in its copy number tend to have a more unfavorable clinical course [50], [51]. In addition, the identification of the partner gene involved in the *MYC* rearrangement is also important, as not all translocations hold the same clinical significance, and translocations involving the *IGH* locus negatively impact prognosis.

Finally, as observed in other hematological neoplasms, *TP53* alterations in DLBCL/HGBCL are linked to poor prognosis and therapy resistance, highlighting the value of *TP53* gene evaluation as a prognostic indicator.

In consideration of the data present in the literature, the aim of the study was to fully characterize a specific subset of DLBCL/HGBCL by integrating various diagnostic methods, obtaining a complete dataset spanning from morphological and immunohistochemical to cytogenetic and molecular data. Finally, by correlating all these data with clinical information, we aimed to stratify patients with high-grade lymphoma and *MYC* gene alterations based on molecularly defined risk.

Regarding the GEP results, the evidence from the literature suggesting that COO GEP is prognostic in unselected cohorts of DLBCL is not reproducible in our cohort, as evidenced by the three-year OS results reported in the 'Results' section. This discrepancy could, in part, be attributed to the sample size and characteristics, and in part explained by the unique characteristics of our cases, highlighting how certain predictors

may be misleading when applied to specific subsets with unique traits. Our cohort was enriched of GCB cases, and this can be particularly explained by the cohort selection. *MYC* translocation, according to literature, albeit present in both GCB and ABC, is more frequent GCB-DLBCL [52]. Overall, the COO data obtained through IHC and GEP were mostly consistent. This concordance rate is consistent with the data reported in the literature [40][53], suggesting that the correlation between GEP and IHC for COO classification is generally reliable, though not without discrepancies in some cases.

Consistent with the results of other studies, MYC-r patients (with or without concomitant copy number gain) had a shorter three-year OS compared to MYC-gain patients, showing a reduction of 52% versus 75%, with a trend toward statistical significance (p=0.19).
In addition, as expected, patients with *MYC* and *BCL2* and/or *BCL6* rearrangements showed a trend to worse OS (3-year OS rate 46%) as compared with patients with *MYC-r* only or with *MYC* gain (3-year OS rate 75% in both groups, p= 0.39). These rearrangements are commonly seen in aggressive lymphomas, and their presence in the cohort highlights the complexity of the molecular landscape.

The *MYC* gene rearrangement in SH, DH, and TH lymphomas is an independent negative prognostic factor, particularly when it involves a *MYC*-IG translocation. In the analyzed cohort, significant differences were observed between the two lymphoma groups with *MYC*-IGH rearrangement vs *MYC*-nonIGH (p=0.02).
This finding strengthens the importance of extending the stratification of individual cases beyond the exclusive evaluation of the presence of the three genetic rearrangements.
Notably, *MYC* copy number gain, regardless of the gene rearrangement, was found in 68% of the analyzed cases (17/25), higher than the frequency reported in the literature, which varies from 7 to 21% in DLBCL [54]. This data is probably influenced by the cohort selection, and secondly by sample size (this data was available only in 25 of the 42 cases analyzed).
The clinical significance and prognostic relevance of numerical *MYC* alterations remain controversial. Recent studies have highlighted an inverse relationship between *MYC* copy number and OS, with worse outcomes observed in patients with >4 copies. Cases with *MYC*>7 or amplification (defined as numerous, unquantifiable signals) showed

particularly poor prognoses, with a median OS of 8 and 8.5 months, respectively [54]. In our cohort, there were no cases with more than 4 gene copies.

In addition, it is well known that *MYC*-driven oncogenesis alone is insufficient to explain the behavioral heterogeneity of high-grade lymphoid neoplasms, and our study's findings strongly underscore this notion.

In our study, the frequency of ***TP53*** mutations in the whole cohort of patients analyzed was 32% (11/34), with 36% (5/14) of mutated cases showing a concomitant genetic deletion. The reported frequencies of *TP53* mutations and mutations associated with deletion in the literature are lower, at 20-25% and 12%, respectively [23]. The higher incidence observed in our study is likely related to the selection criteria of the lymphoma cases, specifically DLBCL/HGBCL with MYC abnormalities, a high proliferative index, and aggressive clinical behavior.
The unique characteristics of the cases included in the study have likely influenced the percentages of co-occurrence of *MYC* rearrangement and *TP53* mutation. It has also been hypothesized that the synchronous *MYC* rearrangement and *TP53* mutation in the same lesion may have a synergistic effect culminating in a lower mean overall survival, comparable to that of DH-MYC/BCL2 lymphomas.
Accordingly, a significant impact on 3-year OS was observed in patients with MYC-r combined with a *TP53* deletion. These cases had a three-year OS of just 20%, a substantial drop when compared to those without *TP53* deletion but with MYC-r (three-year OS 59%) or *MYC* gain (three-year OS 75%) (p=0.047). Considering also MYC-r cases with a concomitant *TP53* mutations, the three-year OS was considerably lower (35%) compared to the other cases (73%) with a statistically significant difference (p=0.016).
This statistically significant difference underscores the negative prognostic implications of *TP53* anomalies (deletions/mutations) in patients with MYC-r, suggesting that *TP53* status could be an important factor to consider in risk stratification and treatment planning for these patients. Thus, by analyzing the mutation status of *TP53*, we can expand the identification of molecularly defined patients with aggressive disease, ensuring a more precise risk stratification.

In addition to *TP53*, in other cases of our cohort with a high aggressive clinical course, *MYC* rearrangements were associated with mutations in the ***CREBBP*** gene and ***MYC*** gene itself.

As already known, somatic mutations in *CREBBP* are among the most common in large B cells lymphoma. Cases classified within the EZB subtype are enriched with this mutation. *CREBBP* encodes a transcriptional coactivator with acetyltransferase activity, being involved in many biological processes such as cell growth, differentiation, stress response, and cell cycle regulation.

Somatic mutations alter acetyltransferase activity inducing constitutive activation of *BCL6* and inhibition *TP53 expression*, thereby conferring a negative impact on prognosis.

Interestingly, *CREBBP* could represent a promising therapeutic target, particularly for treatments aimed at modulating acetylation processes, such as HDAC inhibitors [55].

However, in our cohort, the three-year OS of cases with *CREBBP* mutations was better compared to the other cases, although the difference did not reach statistical significance.

Among the cases with *CREBBP* mutations in our cohort, 11 out of 13 did not harbor a concomitant *TP53* mutation, suggesting that the presence of *TP53* mutations might have a more significant prognostic impact, potentially contributing to the worse prognosis observed in other cases.

Less known are the frequency of *MYC* mutations and their prognostic relevance; most non-synonymous mutations do not appear to have the same adverse prognostic impact as *MYC* translocations.

However, in our study, *MYC* mutations were identified in 31% (13/42) of the analysed cases, and in 61% (8/13) of them were associated with a *TP53* mutation.

Of note, stratifying cases by *MYC* mutations revealed significant prognostic differences, with *MYC*-mutated cases showing reduced overall survival (36% vs. 68%, $p = 0.021$). Conversely, no significant differences were observed when cases were stratified by *MYC* translocations or numerical gains.

These findings further support the importance of mutational profiling in identifying high-risk patients.

These findings confirm the importance of not only determining *MYC*, *BCL2*, and *BCL6* rearrangements: *TP53* mutations/deletions but also additional molecular biomarkers can aid to reach a better risk stratification, reflecting the degree of disease aggressiveness and guide clinicians in selecting targeted therapeutic regimens. Further studies with

larger DLBCL cohorts and different histopathological characteristics will be necessary and will allow for the identification of a greater number of biomarkers, suitable depending on the patient's and disease's characteristics.

As described in the results, the published LymphGen algorithm to a validated clinical NGS assay (TSO500) were applied.
Interestingly, in our cohort the signatures distribution was quite different from literature data about general distribution in large B cell lymphomas.
In fact, the cases that were successfully classified included 34% EZB type, 16% MCD type, 5% ST2 type, 3% N1 type, 3% composite, BN2/N1 type, compared to the original NCI LymphGen study which included 13.9% MCD type, 16.1% BN2 type, 13.2% EZB type, 6.6% A53 type, 4.7% ST2 type, and 2.8% N1 type [36]. The differences observed in the distribution of LymphGen subtypes in our cohort compared to the original NCI study highlight the unique composition of our cohort, which includes exclusively cases with *MYC* alterations. This specificity may have enriched subtypes such as EZB and MCD, while reducing the representation of others. In contrast, the original LymphGen study analyzed a more heterogeneous population of large B-cell lymphomas. These findings emphasize how the composition of the cohort can influence the interpretation of molecular classifications, particularly in specific subsets such as *MYC*-altered cases.
The percentage of cases that remain unassigned is similar in both our cohort and the NCI cohort (37% and 36.9% respectively), which suggests that the assignment of subtypes is reliable, thus supporting the validity of our approach. The feasibility of LymphGen algorithim was also assessed by using a validated clinical NGS assay: the Memorial Sloan Kettering Cancer Center’s (MSKCC) Integrated Mutation Profiling of Actionable Cancer Targets for Hematologic malignancies NGS panel (MSK-IMPACT HEME). The signature distributions were largely consistent with those seen in the NCI study [56]. This alignment supports the robustness of using a target panel for molecular subclassification in DLBCL, confirming that the genetic signatures identified are comparable to those reported using target panel with a similar coverage to our chosen panel.
Stratifying the cohort by mutational signatures, according to LymphGen algorithm, did not identify significant differences, likely due to the sample size.
On the other hand, MCD cases in our cohort showed a trend toward better overall survival compared to other subgroups, in contrast to what previously reported. It should be noted that, compared to the data in the literature, the current study did not include

cases of Primary large B-cell lymphoma of immune-privileged sites such as Primary Central Nervous System Lymphoma. These conditions, sharing some molecular alterations with the MCD signature found in DLBCL NOS, exhibit distinct clinical behavior and may impact the overall OS. Therefore, the observed results for the MCD subgroup should be interpreted with caution, considering the differences in the patient groups analyzed.

These findings further highlight that, in specific cohorts like ours with unique characteristics, canonical predictors may not accurately reflect clinical outcomes.

Conclusions

Based on our data and literature evidence, the heterogeneity of Large B Cell Lymphoma highlights how, even within the same condition, different cohorts with distinct cytogenetic/molecular features can be identified. Our findings clearly demonstrate that the canonical predictors used for prognostic and predictive purposes are insufficient.

This emphasizes the importance of comprehensive profiling in high-risk LBCL patients with *MYC* alterations (structural and/or numerical), going beyond the standard evaluations required for diagnostic definition. This is supported by our cytogenetic and mutational data, where we observed significant differences in overall survival with a more detailed FISH characterization (*MYC* fusion partner and *TP53* deletion) and the evaluation of specific molecular mutations.

In conclusion, additional FISH tests and molecular sequencing provide useful information to the standard diagnostic work-up for DLBCL/HGBL patients thus enabling the refinement of prognostic stratification of patients and guide treatment strategies. This could envision the facilitation of an earlier introduction of novel immunotherapies in dedicated clinical trials.

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Bibliography

[1] R. Alaggio *et al.*, “The 5th edition of the World Health Organization Classification of Haematolymphoid Tumours: Lymphoid Neoplasms,” *Leukemia*, vol. 36, no. 7, pp. 1720–1748, Jul. 2022, doi: 10.1038/s41375-022-01620-2.

[2] E. Campo *et al.*, “The International Consensus Classification of Mature Lymphoid Neoplasms: a report from the Clinical Advisory Committee,” *Blood*, vol. 140, no. 11, pp. 1229–1253, Sep. 2022, doi: 10.1182/blood.2022015851.

[3] R. Küppers, “Mechanisms of B-cell lymphoma pathogenesis,” *Nat Rev Cancer*, vol. 5, no. 4, pp. 251–262, Apr. 2005, doi: 10.1038/nrc1589.

[4] K. R. Shankland, J. O. Armitage, and B. W. Hancock, “Non-Hodgkin lymphoma,” *The Lancet*, vol. 380, no. 9844, pp. 848–857, Sep. 2012, doi: 10.1016/S0140-6736(12)60605-9.

[5] *WHO Classification of Tumours Editorial Board. Haematolymphoid tumours. Lyon (France): International Agency for Research on Cancer; 2024. (WHO classification of tumours series, 5th ed.; vol. 11). https://publications.iarc.who.int/637.*

[6] M. Martelli, A. J. M. Ferreri, C. Agostinelli, A. Di Rocco, M. Pfreundschuh, and S. A. Pileri, “Diffuse large B-cell lymphoma,” *Crit Rev Oncol Hematol*, vol. 87, no. 2, pp. 146–171, Aug. 2013, doi: 10.1016/j.critrevonc.2012.12.009.

[7] S. E. Lacy *et al.*, “Targeted sequencing in DLBCL, molecular subtypes, and outcomes: a Haematological Malignancy Research Network report,” *Blood*, vol. 135, no. 20, pp. 1759–1771, May 2020, doi: 10.1182/blood.2019003535.

[8] A. A. Alizadeh *et al.*, “Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling,” *Nature*, vol. 403, no. 6769, pp. 503–511, Feb. 2000, doi: 10.1038/35000501.

[9] C. P. Hans *et al.*, “Confirmation of the molecular classification of diffuse large B-cell lymphoma by immunohistochemistry using a tissue microarray,” *Blood*, vol. 103, no. 1, pp. 275–282, Jan. 2004, doi: 10.1182/blood-2003-05-1545.

[10] B. Chapuy *et al.*, “Molecular subtypes of diffuse large B cell lymphoma are associated with distinct pathogenic mechanisms and outcomes,” *Nat Med*, vol. 24, no. 5, pp. 679–690, May 2018, doi: 10.1038/s41591-018-0016-8.

[11] S. E. Lacy *et al.*, “Targeted sequencing in DLBCL, molecular subtypes, and outcomes: a Haematological Malignancy Research Network report,” 2020. [Online]. Available: https://www.hmrn.org

[12] K. S. Kurz *et al.*, “Large B-Cell Lymphomas in the 5th Edition of the WHO-Classification of Haematolymphoid Neoplasms—Updated Classification and New

Concepts," *Cancers (Basel)*, vol. 15, no. 8, p. 2285, Apr. 2023, doi: 10.3390/cancers15082285.

[13] C. Y. Ok and L. J. Medeiros, "High-grade B-cell lymphoma: a term re-purposed in the revised WHO classification," *Pathology*, vol. 52, no. 1, pp. 68–77, Jan. 2020, doi: 10.1016/j.pathol.2019.09.008.

[14] D. S. D. A. Salam, E. E. Thit, S. H. Teoh, S. Y. Tan, S. C. Peh, and S.-C. Cheah, "*C-MYC, BCL2* and *BCL6* Translocation in B-cell Non-Hodgkin Lymphoma Cases," *J Cancer*, vol. 11, no. 1, pp. 190–198, 2020, doi: 10.7150/jca.36954.

[15] Y. A. Cho *et al.*, "MYC single-hit large B-cell lymphoma: clinicopathologic difference from MYC-negative large B-cell lymphoma and MYC double-hit/triple-hit lymphoma," *Hum Pathol*, vol. 113, pp. 9–19, Jul. 2021, doi: 10.1016/j.humpath.2021.03.006.

[16] A. Rosenwald *et al.*, "Prognostic Significance of *MYC* Rearrangement and Translocation Partner in Diffuse Large B-Cell Lymphoma: A Study by the Lunenburg Lymphoma Biomarker Consortium," *Journal of Clinical Oncology*, vol. 37, no. 35, pp. 3359–3368, Dec. 2019, doi: 10.1200/JCO.19.00743.

[17] L. C. Chong *et al.*, "High-resolution architecture and partner genes of MYC rearrangements in lymphoma with DLBCL morphology," *Blood Adv*, vol. 2, no. 20, pp. 2755–2765, Oct. 2018, doi: 10.1182/bloodadvances.2018023572.

[18] A. Rosenwald *et al.*, "Prognostic Significance of *MYC* Rearrangement and Translocation Partner in Diffuse Large B-Cell Lymphoma: A Study by the Lunenburg Lymphoma Biomarker Consortium," *Journal of Clinical Oncology*, vol. 37, no. 35, pp. 3359–3368, Dec. 2019, doi: 10.1200/JCO.19.00743.

[19] R. K. Pillai, M. Sathanoori, S. B. Van Oss, and S. H. Swerdlow, "Double-hit B-cell Lymphomas With BCL6 and MYC Translocations Are Aggressive, Frequently Extranodal Lymphomas Distinct From BCL2 Double-hit B-cell Lymphomas," *American Journal of Surgical Pathology*, vol. 37, no. 3, pp. 323–332, Mar. 2013, doi: 10.1097/PAS.0b013e31826cebad.

[20] K. S. Hüttl *et al.*, "The 'Burkitt-like' immunophenotype and genotype is rarely encountered in diffuse large B cell lymphoma and high-grade B cell lymphoma, NOS," *Virchows Archiv*, vol. 479, no. 3, pp. 575–583, Sep. 2021, doi: 10.1007/s00428-021-03050-4.

[21] S. Huang *et al.*, "Prognostic impact of diffuse large B-cell lymphoma with extra copies of MYC, BCL2 and/or BCL6: comparison with double/triple hit lymphoma and double expressor lymphoma," *Diagn Pathol*, vol. 14, no. 1, p. 81, Dec. 2019, doi: 10.1186/s13000-019-0856-7.

[22] E. N. Voropaeva, T. I. Pospelova, M. I. Voevoda, V. N. Maksimov, Y. L. Orlov, and O. B. Seregina, "Clinical aspects of TP53 gene inactivation in diffuse large B-cell

lymphoma," *BMC Med Genomics*, vol. 12, no. S2, p. 35, Mar. 2019, doi: 10.1186/s12920-019-0484-9.

[23] Z. Y. Xu-Monette *et al.*, "Mutational profile and prognostic significance of TP53 in diffuse large B-cell lymphoma patients treated with R-CHOP: report from an International DLBCL Rituximab-CHOP Consortium Program Study," *Blood*, vol. 120, no. 19, pp. 3986–3996, Nov. 2012, doi: 10.1182/blood-2012-05-433334.

[24] K. H. Young *et al.*, "Structural profiles of TP53 gene mutations predict clinical outcome in diffuse large B-cell lymphoma: an international collaborative study," *Blood*, vol. 112, no. 8, pp. 3088–3098, Oct. 2008, doi: 10.1182/blood-2008-01-129783.

[25] S. Sangaletti *et al.*, "Intra-tumour heterogeneity of diffuse large B-cell lymphoma involves the induction of diversified stroma-tumour interfaces," *EBioMedicine*, vol. 61, p. 103055, Nov. 2020, doi: 10.1016/j.ebiom.2020.103055.

[26] S. Heim and F. Mitelman, Eds., *Cancer Cytogenetics*. Wiley, 2015. doi: 10.1002/9781118795569.

[27] H. Al-Maghrabi and J. Al-Maghrabi, "Composite Lymphoma's Ability to Adapt and Change Through Plasticity: A Case Report and Literature Review," *Cureus*, Sep. 2023, doi: 10.7759/cureus.45696.

[28] A. M. Moore, O. Moshkin, G. J. Swain, S. Crocker, and D. P. LeBrun, "High-grade B-cell lymphoma with MYC and BCL2 rearrangements arising in a composite lymphoma," *Diagn Pathol*, vol. 13, no. 1, p. 34, Dec. 2018, doi: 10.1186/s13000-018-0714-z.

[29] C. P. Hans, "Confirmation of the molecular classification of diffuse large B-cell lymphoma by immunohistochemistry using a tissue microarray," *Blood*, vol. 103, no. 1, pp. 275–282, Jan. 2004, doi: 10.1182/blood-2003-05-1545.

[30] W. W. L. Choi *et al.*, "A New Immunostain Algorithm Classifies Diffuse Large B-Cell Lymphoma into Molecular Subtypes with High Accuracy," *Clinical Cancer Research*, vol. 15, no. 17, pp. 5494–5502, Sep. 2009, doi: 10.1158/1078-0432.CCR-09-0113.

[31] P. N. Meyer *et al.*, "Immunohistochemical Methods for Predicting Cell of Origin and Survival in Patients With Diffuse Large B-Cell Lymphoma Treated With Rituximab," *Journal of Clinical Oncology*, vol. 29, no. 2, pp. 200–207, Jan. 2011, doi: 10.1200/JCO.2010.30.0368.

[32] T. M. Green *et al.*, "Immunohistochemical Double-Hit Score Is a Strong Predictor of Outcome in Patients With Diffuse Large B-Cell Lymphoma Treated With Rituximab Plus Cyclophosphamide, Doxorubicin, Vincristine, and Prednisone," *Journal of Clinical Oncology*, vol. 30, no. 28, pp. 3460–3467, Oct. 2012, doi: 10.1200/JCO.2011.41.4342.

[33] R. M. Young and L. M. Staudt, “Targeting pathological B cell receptor signalling in lymphoid malignancies,” *Nat Rev Drug Discov*, vol. 12, no. 3, pp. 229–243, Mar. 2013, doi: 10.1038/nrd3937.

[34] Y. Jiang, P. M. Dominguez, and A. M. Melnick, “The many layers of epigenetic dysfunction in B-cell lymphomas,” *Curr Opin Hematol*, vol. 23, no. 4, pp. 377–384, Jul. 2016, doi: 10.1097/MOH.0000000000000249.

[35] R. Schmitz *et al.*, “Genetics and Pathogenesis of Diffuse Large B-Cell Lymphoma,” *New England Journal of Medicine*, vol. 378, no. 15, pp. 1396–1407, Apr. 2018, doi: 10.1056/nejmoa1801445.

[36] G. W. Wright *et al.*, “A Probabilistic Classification Tool for Genetic Subtypes of Diffuse Large B Cell Lymphoma with Therapeutic Implications,” *Cancer Cell*, vol. 37, no. 4, pp. 551-568.e14, Apr. 2020, doi: 10.1016/j.ccell.2020.03.015.

[37] R. D. Morin, S. E. Arthur, and D. J. Hodson, “Molecular profiling in diffuse large B-cell lymphoma: why so many types of subtypes?,” Feb. 01, 2022, *John Wiley and Sons Inc*. doi: 10.1111/bjh.17811.

[38] Z. A. K. Frosch and D. J. Landsburg, “Molecular Risk Stratification in Aggressive B-Cell Lymphomas,” *Journal of Clinical Oncology*, vol. 38, no. 18, pp. 2014–2017, Jun. 2020, doi: 10.1200/JCO.19.03069.

[39] P. Farinha *et al.*, “TP53 Expression Correlates with TP53 Mutations and Is an Independent Predictor of Clinical Outcome in Patients with DLBCL Treated with R-CHOP,” *Blood*, vol. 134, no. Supplement_1, pp. 3964–3964, Nov. 2019, doi: 10.1182/blood-2019-121943.

[40] M. Abdulla *et al.*, “Cell-of-origin determined by both gene expression profiling and immunohistochemistry is the strongest predictor of survival in patients with diffuse large B-cell lymphoma,” *Am J Hematol*, vol. 95, no. 1, pp. 57–67, Jan. 2020, doi: 10.1002/ajh.25666.

[41] L. C. Chong *et al.*, “High-resolution architecture and partner genes of MYC rearrangements in lymphoma with DLBCL morphology,” *Blood Adv*, vol. 2, no. 20, pp. 2755–2765, Oct. 2018, doi: 10.1182/bloodadvances.2018023572.

[42] R. J. Hastings *et al.*, “Guidelines for cytogenetic investigations in tumours,” *European Journal of Human Genetics*, vol. 24, no. 1, pp. 6–13, Jan. 2016, doi: 10.1038/ejhg.2015.35.

[43] P. A. Riedell and S. M. Smith, “Double hit and double expressors in lymphoma: Definition and treatment,” *Cancer*, vol. 124, no. 24, pp. 4622–4632, Dec. 2018, doi: 10.1002/cncr.31646.

[44] Y. W. Koh, H. S. Hwang, C. S. Park, D. H. Yoon, C. Suh, and J. Huh, “Prognostic effect of Ki-67 expression in rituximab, cyclophosphamide, doxorubicin, vincristine

and prednisone-treated diffuse large B-cell lymphoma is limited to non-germinal center B-cell-like subtype in late-elderly patients," *Leuk Lymphoma*, vol. 56, no. 9, pp. 2630–2636, Sep. 2015, doi: 10.3109/10428194.2015.1004169.

[45] A. Rosenwald *et al.*, "Prognostic Significance of *MYC* Rearrangement and Translocation Partner in Diffuse Large B-Cell Lymphoma: A Study by the Lunenburg Lymphoma Biomarker Consortium," *Journal of Clinical Oncology*, vol. 37, no. 35, pp. 3359–3368, Dec. 2019, doi: 10.1200/JCO.19.00743.

[46] A. Clipson *et al.*, "The prognosis of *MYC* translocation positive diffuse large B-cell lymphoma depends on the second hit," *J Pathol Clin Res*, vol. 1, no. 3, pp. 125–133, Jul. 2015, doi: 10.1002/cjp2.10.

[47] X. Jiang *et al.*, "Subtyping Diffuse Large B-Cell Lymphoma with Cell-of-Origin Using 32-Gene Expression Assay in Formalin-Fixed Paraffin-Embedded Tissue," *Blood*, vol. 134, no. Supplement_1, pp. 5337–5337, Nov. 2019, doi: 10.1182/blood-2019-126629.

[48] A. Cabal *et al.*, "LymphGen Classification of Diffuse Large B-Cell Lymphoma in a Cohort of Adolescent and Young Adult (AYA) Patients," *Blood*, vol. 140, no. Supplement 1, pp. 6392–6393, Nov. 2022, doi: 10.1182/blood-2022-162418.

[49] M. Laude *et al.*, "First-line treatment of double-hit and triple-hit lymphomas: Survival and tolerance data from a retrospective multicenter French study," *Am J Hematol*, vol. 96, no. 3, pp. 302–311, Mar. 2021, doi: 10.1002/ajh.26068.

[50] C. J. Stasik *et al.*, "Increased MYC gene copy number correlates with increased mRNA levels in diffuse large B-cell lymphoma," *Haematologica*, vol. 95, no. 4, pp. 597–603, Apr. 2010, doi: 10.3324/haematol.2009.012864.

[51] S. Huang *et al.*, "Prognostic impact of diffuse large B-cell lymphoma with extra copies of MYC, BCL2 and/or BCL6: comparison with double/triple hit lymphoma and double expressor lymphoma," *Diagn Pathol*, vol. 14, no. 1, p. 81, Dec. 2019, doi: 10.1186/s13000-019-0856-7.

[52] A. Rosenwald *et al.*, "Prognostic Significance of *MYC* Rearrangement and Translocation Partner in Diffuse Large B-Cell Lymphoma: A Study by the Lunenburg Lymphoma Biomarker Consortium," *Journal of Clinical Oncology*, vol. 37, no. 35, pp. 3359–3368, Dec. 2019, doi: 10.1200/JCO.19.00743.

[53] R. Crotty *et al.*, "Simultaneous Identification of Cell of Origin, Translocations, and Hotspot Mutations in Diffuse Large B-Cell Lymphoma Using a Single RNA-Sequencing Assay," *Am J Clin Pathol*, vol. 155, no. 5, pp. 748–754, May 2021, doi: 10.1093/ajcp/aqaa185.

[54] F. Schieppati *et al.*, "An increase in *MYC* copy number has a progressive negative prognostic impact in patients with diffuse large B-cell and high-grade lymphoma,

who may benefit from intensified treatment regimens," *Haematologica*, vol. 105, no. 5, pp. 1369–1378, May 2020, doi: 10.3324/haematol.2019.223891.

[55] I.-C. Chen, B. Sethy, and J.-P. Liou, "Recent Update of HDAC Inhibitors in Lymphoma," *Front Cell Dev Biol*, vol. 8, Sep. 2020, doi: 10.3389/fcell.2020.576391.

[56] M.-L. Zhu *et al.*, "Validation of LymphGen classification on a 400-gene clinical next-generation sequencing panel in diffuse large B-cell lymphoma: real-world experience from a cancer center," *Haematologica*, Mar. 2024, doi: 10.3324/haematol.2023.284565.