Predictive and Prognostic Molecular Factors in Diffuse Large B-Cell Lymphomas

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Abstract: Diffuse large B-cell lymphoma (DLBCL) is the most common form of lymphoid malignancy, with a prevalence of about 40% worldwide. Its classification encompasses a common form, also termed as “not otherwise specified” (NOS), and a series of variants, which are rare and at least in part related to viral agents. Over the last two decades, DLBCL-NOS, which accounts for more than 80% of the neoplasms included in the DLBCL chapter, has been the object of an increasing number of molecular studies which have led to the identification of prognostic/predictive factors that are increasingly entering daily practice. In this review, the main achievements obtained by gene expression profiling (with respect to both neoplastic cells and the microenvironment) and next-generation sequencing will be discussed and compared. Only the amalgamation of molecular attributes will lead to the achievement of the long-term goal of using tailored therapies and possibly chemotherapy-free protocols capable of curing most (if not all) patients with minimal or no toxic effects.

Keywords: diffuse large B-cell lymphoma; gene expression profiling; next-generation sequencing; classification; diagnosis; prognosis; therapy

1. Classification

Diffuse large B-cell lymphoma (DLBCL) is the most common form of lymphoid malignancy, with a prevalence of about 40% worldwide [1]. It consists of medium or large B-lymphoid cells in which the nuclei are the same size as or larger than those of normal macrophages, or more than twice the size of those of normal lymphocytes, with a diffuse growth pattern [1]. The concept of DLBCL has undergone fine-tuning over time, as is clear from the comparisons between the REAL and WHO classifications (third, fourth, and revised fourth editions) [1–4]. This produces some apparent terminological discrepancies throughout the text, which reflect the time of publication of each reference.

In the Revised Fourth Edition of the WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues, DLBCL is subdivided into morphologic variants, molecular subtypes, and distinct disease entities (Table 1) [1]. Nevertheless, about 70% of all DLBCLs lack features allowing their inclusion into one of the diagnostic categories listed in Table 1 [1]. These cases are collectively termed as “not otherwise specified” (DLBCL-NOS) [1] and are conventionally treated with the chemoimmunotherapy regimen R-CHOP [5,6].
Table 1. Diffuse large B-cell lymphoma, high-grade B-cell lymphoma, and gray-zone lymphoma according to the Revised Fourth Edition of the WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues (italics indicate that an entity is provisional).

| Diffuse Large B-Cell Lymphoma (DLBCL): |
|-----------------------------------------|
| DLBCL not otherwise specified (NOS)    |
| Morphological variants                 |
| Centroblastic                          |
| Immunoblastic                          |
| Anaplastic                             |
| Other rare variants                    |
| Molecular subtypes                     |
| Germinal centre B-cell subtype (GCB)   |
| Activated B-cell subtype (ABC)         |

| Other lymphomas of Large B-Cells:  |
|----------------------------------|
| T-cell/histiocyte-rich large B-cell lymphoma |
| Primary DLBCL of the CNS          |
| Primary cutaneous DLBCL, leg type  |
| EBV-positive DLBCL, NOS           |
| EBV-positive mucocutaneous ulcer  |
| DLBCL associated with chronic inflammation |
| Lymphomatoid granulomatosis      |
| Large B-cell lymphoma with IRF4 rearrangement |
| Primary mediastinal (thymic) large B-cell lymphoma |
| Intravascular large B-cell lymphoma |
| ALK-positive large B-cell lymphoma |
| Plasmablastic lymphoma           |
| HHV8-positive DLBCL              |
| Primary effusion lymphoma        |

| High-Grade B-Cell Lymphoma:        |
|-----------------------------------|
| High-grade B-cell lymphoma with MYC and BCL2 and/or BCL6 rearrangement |
| High-grade B-cell lymphoma, not otherwise specified (NOS) |

B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and classic Hodgkin’s lymphoma

Based on a recent survey of 3550 DLBCL patients who mostly underwent R-CHOP with curative intent, the 5-year overall survival and cumulative incidence of relapsed/refractory disease corresponds to 65.3% and 23.1% of cases, respectively [6]. Thus, there is still an unmet need for optimal therapy for a significant proportion of DLBCL-NOS patients.

In recent years, DLBCL-NOS has been the object of the extensive application of high-throughput technologies, which has led to the identification of prognostic/predictive factors that are increasingly entering daily practice.

Although DLBCL-NOS is the main focus of this review, the borders between DLBCL-NOS and high-grade B-cell lymphoma (HGBCL) (Table 1) will also be discussed. In fact, it is not uncommon to encounter cases that could be regarded as DLBCL-NOS but are ultimately classified as HGBCL due to the detection of double or triple hits (D/TH) of MYC, BCL2, and/or BCL6 (HGBCL-D/TH) by FISH, as underlined by Sehn and Salles.
in their review on DLBCL published in the *New England Journal of Medicine* on 4 March 2021 [7] (see below).

2. Gene Expression Profiling

2.1. Cell of Origin (COO)

At the beginning of this century, using gene expression profiling (GEP) Alizadeh and coworkers first reported that DLBCLs could be divided into two main subtypes with a gene signature related to the germinal center B-cell (GCB) and activated B-lymphocytes from the peripheral blood (ABC), respectively [8]. Such a distinction, not feasible on morphological grounds, had an important prognostic impact. In fact, the GCB forms had a significantly more favorable response to chemotherapy (CHOP) than those of ABC. This corresponded to a clear-cut difference in terms of overall and progression-free survival (OS and PFS, respectively). This subdivision was subsequently confirmed using cohorts consisting of hundreds of cases, and maintained its value in the era of chemoimmunotherapy [9–11]. By expanding the number of profiled cases, a third group between those of GCB and ABC emerged and was indicated as unclassified (U), corresponding to about 15% of DLBCLs [9–11]. Besides prognostic value, the distinction between GCB and ABC subtypes has biological relevance as it corresponds to different genetic aberrations as well as pathway perturbations (as detailed in the following).

The main limitation of conventional GEP was the need for fresh or frozen (FF) samples, which were available for a small minority of patients followed up at reference centers. Therefore, many attempts were made to find surrogates for GEP through the search for immunohistochemical markers [12–18]. Several algorithms were proposed, with that of Hans et al. having the widest applications as it was based on the simple determination of CD10, BCL6, and IRF4/MUM1 [12]. However, none of these algorithms met their goal, for several reasons: (a) a lack of correspondence with GEP data in the same patients; (b) variability in the preanalytical and immunohistochemical techniques (including antibody and antigen retrieval, detection systems, and automatic platforms); and (c) subjectivity in result interpretation [19,20].

In 2014, a new approach was proposed based on targeted digital GEPFF and was successfully applied to mRNA extracted from formalin-fixed, paraffin-embedded (FFPE) tissue samples (Lymph2Cx) [21]. In particular, a 20-gene panel (including 15 top genes and 5 housekeeping genes for normalization) was designed, which in 67 cases provided the same COO classification as conventional GEP from FF. Furthermore, the OS and PFS curves were over-imposable, irrespective of the type of GEP used (targeted digital vs. conventional). These preliminary results, which had been obtained by using the NanoString platform, were subsequently confirmed by independent studies based on several hundred cases [22–25]. The advantages of this approach over immunohistochemical algorithms are: (1) reproducibility in different laboratories; (2) the assessment of the absolute value of mRNA expressed by each gene; and (3) a lack of confounding factors (such as the variability of immunohistochemical techniques and subjective result interpretation). Moreover, targeted GEP subdivides DLBCL-NOS into GCB, ABC, and U, like conventional profiling of FF samples. In contrast, immunohistochemical algorithms differentiate DLBCL-NOS into GCB and non-GCB, with the latter group containing cases that are molecularly classified as GCB [21–25]. Interestingly, identical results were obtained by targeted profiling on different platforms and with different panels of genes confirming the prognostic relevance of the COO determination [21–25].

The COO determination provided less significant prognostic information when applied to cases enrolled in some trials [26–28]. This may be for several different reasons, i.e., (1) the adoption of protocols that are more intense than those used in real situations; (2) the selection of patients fit enough to await the completion of all the tests required for trial enrolment; and (3) the influence of other factors that can affect behavior within each subgroup defined by the COO.
The main limitation of targeted GEP applied to routine biopsies is the need for platforms which are not available in all pathology laboratories, unlike immunohistochemistry. This problem, as well as the test costs and need for basic bioinformatic skills, can be overcome by a hub-and-spoke organization, which is also required for the application of the array of molecular techniques at the basis of precision medicine (see below).

2.2. Key Genes

FISH analyses have shown that B-cell lymphomas, regarded as DLBCLs-NOS based on morphology and phenotype, could carry double or triple rearrangements of MYC, BCL2, and/or BCL6 [1]. These cases, which overall have a significantly worse prognosis and may require therapies that are more intense than standard R-CHOP, are nowadays included in the provisional category of high-grade B-cell lymphomas with double/triple hits (HGBL D/TH). Based on this observation, FISH should ideally be applied to all DLBCL-NOS cases. As FISH analyses are rather expensive, attempts have been made to find surrogates for FISH results through immunohistochemistry. This has led to the identification of a group of DLBCL-NOS cases which show double expression of MYC and BCL2 at the protein level (the so-called double expressors (DEs)) [29,30]. According to the Revised Fourth Edition of the WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues, at least 50% and 40% of neoplastic cells should express BCL2 and MYC, respectively, in order to consider a DLBCL-NOS patient as a DE [1]. However, discrepancies exist as to the reproducibility of the cut-off value of MYC positivity, which has been moved to 70% by some groups [31]. Most importantly, as there is no actual correspondence between the results of immunohistochemistry and FISH [32], these cases with MYC and BCL2 double-expression but lacking D/TH remain within the bounds of DLBCL-NOS but more often belong to the ABC/non-GCB subtype and require further studies to definitively assess their prognostic and/or therapeutic relevance [1,32].

In 2018, investigators from two groups used GEP signatures to identify high-risk patients with DLBCL in FFPE series. Sha et al. [33] used a Burkitt lymphoma-like signature, whereas Ennishi et al. [34] used a signature derived from genes differentially expressed between MYC/BCL2 DH and non-DH GCB-DLCLs. With their respective signatures, these investigators were, as expected, able to identify most DH lymphomas, as well as many non-DH lymphomas which were actually found in about half of the identified patients and showed a poorer response to standard chemoimmunotherapy. These findings suggest that many of the patients harbored genetic or even epigenetic alterations that produced similar gene expression changes in the tumor cells, as recognized by the respective signatures. This does not come as a surprise. In fact, activation of an oncogene or oncogenic pathway can be produced by multiple mechanisms besides MYC/BCL2 DH, for example MYC upregulation through translocation and gene amplification. Apart from structural alterations, MYC expression or activity can be enhanced through transcriptional and posttranscriptional events.

Derenzini et al. [25] used a targeted GEP panel combining the Lymph2Cx signature for COO classification, with additional targets including MYC, BCL-2, and NFKBIA (the latter encoding for the IκB-α protein, an endogenous inhibitor of NF-kβ signaling [35]), in 186 FFPE cases originally diagnosed as DLBCL-NOS from two randomized trials (discovery cohorts NCT0355199 and NCT00499018) and in three independent validation cohorts. By integrating the COO, MYC/BCL-2 DE status, and NFKBIA expression, a three-gene signature was designed combining MYC, BCL-2, and NFKBIA (MBN signature). The high-risk (MBN Sig-high) subgroup, characterized by higher expression levels of MYC and BCL-2 and a lower expression of NFKBIA, could be used to identify a significant fraction of ABC DLBCLs and the vast majority of double-hit cases, allowing for further risk stratification within the GCB/U subset. These results were validated in three independent series including Sha’s cohort based on the REMoDL-B trial [33,36], a phase III randomized trial investigating the efficacy of the addition of bortezomib to standard first-line chemoimmunotherapy. In line with the biological activity of bortezomib, which increases the protein abundance of IκB-α, leading to inhibition of NF-kB signaling [37], an exploratory ad hoc analysis of the latter cohort showed that the addition of bortezomib in the MBN Sig-high subgroup provided
a progression-free survival advantage compared with standard chemoimmunotherapy. These data suggest that a simple three-gene signature based on MYC, BCL-2, and NFKBIA can refine the prognostic stratification in DLBCL.

Finally, Mottok et al. [38] developed a robust and accurate molecular classification assay (Lymph3Cx) for the distinction of primary mediastinal B-cell lymphoma (PMBCL) from DLBCL subtypes based on gene expression measurements in formalin-fixed, paraffin-embedded tissue. A probabilistic model accounting for classification error comprising 58 gene features was trained on 68 cases of PMBCL and DLBCL. Model performance was subsequently evaluated in an independent validation cohort of 158 cases and showed a high agreement of the Lymph3Cx molecular classification with the clinicopathological diagnosis of an expert panel (frank misclassification rate, 3.8%). In the authors’ view, Lymph3Cx represents a molecular tool that is potentially helpful for the diagnosis of PMBCL in light of the use of ad hoc therapeutic approaches [1]. In fact, on central review, cases enrolled in trials as DLBCL-NOS are not infrequently reclassified as PMBCL and vice versa, as seen in the authors’ experience at a reference center for several trials of the Italian Lymphoma Foundation.

3. Tissue Microenvironment (TME)

By means of a gene profiling analysis of nearly 500 FF DLBCL samples, in 2008 Lenz et al. first demonstrated that the expression of peculiar gene sets, namely “Stromal-1” and “Stromal-2” signatures, respectively correlated with good- and poor-outcome subgroups of R-CHOP-treated patients independently of COO [39]. Although they were selectively enriched in genes encoding extracellular matrix proteins and histiocyte infiltration (Stromal-1) or reflecting angiogenesis (Stromal-2), these signatures resulted mechanistically uninformative, and their practical use was limited by the lack of standardized GEP assays for FFPE samples. A number of subsequent research attempts were aimed at identifying TME-related prognostic factors, but none provided biomarkers reproducible enough to be translated into daily clinical practice [40–43].

To overcome this limitation, in 2018 Ciavarella and co-workers [44] generated a 1028-gene matrix incorporating the signatures of 17 cytotypes and applied the computational method CIBERSORT to deconvolve Lenz’s GEP dataset. The work clarified the prognostic associations between patient outcome and quantitative proportions of tumor-infiltrating cell types. A panel of 45 genes related to myofibroblasts (MFs), dendritic cells, and CD4+ T-cells was selected and digitally validated by a NanoString-based approach on an independent cohort of 175 FFPE DLBCLs from two randomized trials. All tissue samples consisted of pretreatment biopsies of advanced-stage nodal DLBCLs treated by comparable R-CHOP/R-CHOP-like regimens. The expression of the 45 TME genes positively correlated with better outcomes and predicted the patient risk of overall and progression-free survival. In a multivariate Cox model, the TME panel retained high prognostic performance independently of COO, and integration of the two prognostic factors (COO + TME) improved survival prediction. Finally, a model to assign single DLBCL cases to a “COO–TME” risk category was built and successfully applied to an independent cohort of 40 “real-life” cases.

In a parallel work, Staiger et al. [45] proposed a lymphoma-associated macrophage interaction signature (LAMIS) interrogating features of the microenvironment, once again using a NanoString assay applicable to FFPE. The clinical impact of the signature was validated in a cohort of 466 patients enrolled in prospective clinical trials at the German High-Grade Non-Hodgkin Lymphoma Study Group (DSHNHL). Patients with high expression of the signature (LAMIS-high) had shorter event-free survival (EFS), progression-free survival (PFS), and overall survival (OS). Multivariate analyses revealed independence from International Prognostic Index (IPI) factors in EFS (HR 1.7, 95%CI 1.2–2.4, p-value = 0.001), PFS (HR 1.8, 95%CI 1.2–2.5, p-value = 0.001), and OS (HR 1.8, 95%CI 1.3–2.7, p-value = 0.001). Multivariate analyses adjusted for the IPI factors showed the signature was independent of COO, MYC rearrangements, and double-expressor (DE) status. LAMIS-high and simultaneous DE status characterized a patient subgroup with dismal prognosis and greater probability of early relapse.
Beyond the prognostic value of TME, only a few studies have provided comprehensive biological insights into the putative link between B-cell genomics and functional patterns of immune and stromal components while suggesting new rationales for future therapeutic approaches.

Tripodo et al. [46] reported on a spatially resolved 53-gene signature comprising key genes of the dark-zone (DZ) mutational machinery, and light-zone (LZ) immune and mesenchymal milieu. This signature was applied to the transcriptomes of 543 cases of GCB-DLBCL and HGBCL-DH. According to the DZ/LZ signature, the GC-related lymphomas were sub-classified into two clusters. The subgroups differed in the distribution of DH cases and survival, with most DH cases displaying a distinct DZ-like profile. The clustering analysis was also performed using a 25-gene signature composed of DZ/LZ genes positively enriched in the non-B, stromal sub-compartments, for the first time achieving DZ/LZ discrimination based on stromal/immune features. The report offers new insight into the GC microenvironment, hinting at a DZ microenvironment of origin in DH lymphomas.

Intriguing research integrating transcriptomic, genetic, and immunophenotypic data of 347 DLBCLs demonstrated that MHC loss, particularly in GC-derived tumors originating from the centroblast-rich DZ, is associated with a strong enrichment of EZH2 mutations, lower T cell infiltration, and poorer outcome [47]. Such results paved the way for the potential use of EZH2 inhibitors to treat the tumor by simultaneously modulating its immune microenvironment.

Finally, very recent work by Kotlov et al. [48] provided a relevant classification of DLBCL based on the transcriptomic characterization of TME from 4655 cases. Four major TME categories were identified as being associated with peculiar genetic/epigenetic aberrations of the malignant component, clinical behavior, and potential therapeutic targeting. Beyond its classification value, to date this work represents the most extensive translational and biological analysis of malignant and non-malignant DLBCL components.

By all means, in-depth TME analysis still represents an approach that can significantly improve the prognostication of DLBCL and even further tune the identification of different risk groups within the same COO category, predicting the response to targeted therapies.

4. Genetic Classification

Over the last few years, several proposals for a genetic classification of DLBCL have been published. Hereunder, the main contributions will be summarized and discussed based on the technical approach used.

4.1. Whole-Exome Sequencing (WES)-Based Studies

In 2017, Reddy and coworkers [49] reported on the whole-exome sequencing (WES) of 1001 FF DLBCLs and 400 paired germline DNAs. They found 150 driver genes to be recurrently mutated. The 60 top genes frequently exhibited a pattern of either predominant missense and/or copy number gains consistent with an oncogene or truncating mutations and/or copy number losses consistent with a tumor suppressor gene. When the mutational pattern was matched with the COO, 20 genes were differentially mutated between the two groups, including EZH2, SGK1, GNA13, SOCS1, STAT6, and TNFRSF14, which were mutated in GCB tumors, and ETV6, MYD88, PIM1 and TBL1XR1, which were mutated in ABC tumors. Interestingly, MLL2 mutations were associated with those of MYC, while TP53 mutations occurred in a mutually exclusive fashion with KLHL6. CRISPR screening revealed that knockout of EBF1, IRF4, CARD11, MYD88, and IKBKB was selectively lethal in ABC DLBCL cell lines, as was knockout of ZBTB7A, XPO1, TGFB2, and PTPN6 in the GCB lines. On prognostic grounds, MYC mutations were strongly associated with poorer survival, as were mutations in CD79B and ZFAT. Mutations in NF1 and SIK1 were associated with more favorable survival. Furthermore, in ABC DLBCLs, genetic alterations in KLHL14, BTG1, PAX5, and CDKN2A were associated with significantly poorer survival, while those in CREBBP were associated with favorable outcomes. In the GCB-DLBCL group, genetic alterations in NFkBIA and NCOR1 were associated with poorer prognosis, while alterations in EZH2, MYD88, and ARID5B were all associated with a significantly better prognosis. The authors developed a multivariate supervised learning approach for
defining the association of survival with combinations of genetic markers (150 genetic driver genes) and gene expression markers (cell of origin, MYC, and BCL2). This led to the proposal of a three-subgroup molecular risk model that was found to outperform all existing predictors (i.e., COO, MYC/BCL2 DE, and IPI). However, the recent application of this model to 499 DLBCLs by Bolen et al. [50] did not provide independent validation. This might reflect the technical differences between the two studies (WES of FF samples by Reddy et al. vs. targeted NGS of DNA extracted from FFPE biopsies by Bolen et al.).

Two studies published in 2018 proposed a molecular subclassification of DLBCLs that had potential prognostic and therapeutic implications [51,52]. They both were based on WES and copy-number analysis of a large series of FF DLBCLs (304 and 574, respectively) [51,52].

Chapuy et al. [51] described five clusters characterized by different genetic lesions that were capable of identifying subgroups within the COO categories showing different behaviors. Most cases included in clusters (Cs) 1 and 5 were classified as ABC. However, they showed important differences on molecular and prognostic grounds. C1 cases were thought to derive from marginal-zone B-cells, as they showed a stable mutational pattern, structural variants (SVs) of BCL6, and mutations of genes involved in the NOTCH2 and NF-kB pathways (NOTCH2, SPEN, BCL10, TNFAIP3, and FAS). Besides the multiple genetic lesions of genes involved in immune escape (BMD2, CD70, FAS, PD-L1, PD-L2), these C1 cases carried MYD88 mutations which were non-L265P, unlike what was observed in the cases included in C5. Notably, C1 cases had a rather favorable course and revealed potential therapeutic targets related to NOTCH2 and BCL6 signaling and immune evasion mechanisms. C5 tumors, which behaved more aggressively than the C1 ones, showed mutations of MYD88<sup>L265P</sup>, CD79B, PIM1, TBL1XR1, GRHPR, and BTKI, SV of 18q, and activation of the NF-kB pathway. In addition, they carried ongoing mutations, being at least in part under the effect of AID. Potential targets for C5 cases corresponded to BCR/TLR signaling and BCL2.

Cs 3 and 4 were significantly enriched in GCB cases but were characterized by different genetic lesions and responses to chemoimmunotherapy. The majority of DLBCLs in C3 harbored BCL2 mutations with concordant SVs. They also exhibited frequent mutations in chromatin modifiers, KMT2D, CREBBP, and EZH2, and increased transcriptional abundance of EZH2 targets by gene set enrichment analyses (GSEA). These tumors also had alterations in the B-cell transcription factors MEF2B and IRF8, and indirect modifiers of BCR and PI3K signaling (TNFSF14(HVEM), HCNV1, and GNA13). In addition, C3 tumors had two alternative mechanisms of inactivating PTEN: focal 10q23.31/PTEN loss and predominantly truncating PTEN mutations, events that play a role in the process of lymphomagenesis. C4 DLBCLs were characterized by mutations in four linker and four core histone genes, multiple immune evasion molecules (CD83, CD58, and CD70), BCR/PI3K signaling intermediates (RHOA, GNA13, and SGK1), NF-kB modifiers (CARD11, NFKBIE, and NFKBIA), and RAS/JAK/STAT pathway members (BRAF and STAT3). Comparison of the C3 and C4 genetic signatures further revealed that these GCB-DLBCLs utilized distinct mechanisms to perturb common pathways such as PI3K signaling. In contrast to C3 DLBCLs, C4 tumors rarely exhibited PTEN alterations but harbored more frequent RHOA mutations. In addition, C4 DLBCLs rarely exhibited BCL2 alterations and had higher mutational density. The distinct genetic features of C3 and C4 GCB-DLBCLs led Chapuy et al. to suggest specific targeted therapies including inhibition of BCL2, PI3K, and the epigenetic modifiers EZH2 and CREBBP in C3 GCB tumors, and JAK/STAT and BRAF/MEK1 blockade in C4 GCB-DLBCLs. Last but not least, C3 cases had a far worse prognosis.

C2 DLBCLs harbored frequent biallelic inactivation of TP53 by mutations and 17p copy loss. In addition, they often exhibited copy loss of 9p21.13/CDKN2A and 13q14.2/RB1, perturbing chromosomal stability and cell cycle. C2 tumors also had significantly more driver somatic copy number alterations (SCNAs) and a higher proportion of genome doubling events. This cluster included both GCB- and ABC-DLBCLs, as did prior DLBCL cohorts with TP53 mutations in targeted analyses [53]. Prognostically significant SCNAs, including 13q31.31/miR-17-92 copy gain and 1q42.12 copy loss, were also more common in these DLBCLs, which were characterized by a rather unfavorable prognosis.
A further cluster, termed 0, was also detected, which apparently lacked significant genetic alterations. However, as the C0 group consisted almost exclusively of T-cell rich/histiocyte-rich B-cell lymphomas, the obtained results might have been largely influenced by the small number of neoplastic cells.

The authors further evaluated BCL2 and MYC alterations. Tumors with cooccurring BCL2 and MYC SVs were significantly more frequent in C3 DLBCLs.

Importantly, the coordinate genetic signatures reported by Chapuy et al. predicted outcomes independent of IPI which could suggest new combination treatment strategies and, more broadly, provide a roadmap for actionable DLBCL classification [51].

By their integrated approach, Schmitz et al. [52] identified four prominent genetic subtypes among 574 DLBCLs which they termed MCD (based on the co-occurrence of MYD88L265P and CD79B mutations), BN2 (based on BCL6 fusions and NOTCH2 mutations), N1 (based on NOTCH1 mutations), and EZB (based on EZH2 mutations and BCL2 translocations). Interestingly, Schmitz and co-workers enriched their series with unclassified DLBCLs. The latter turned out to frequently carry mutations affecting SPEN and NOTCH2 as well as BCL6 fusions. ABC cases were enriched in MYD88L265P and CD79B or NOTCH1 mutations, with the two conditions being mutually exclusive. GCB tumors showed the co-occurrence of EZH2 mutations and BCL2 translocations. The MCD and N1 subtypes were dominated by ABC cases, while EZB included mostly GCB tumors, and BN2 had contributions from all GEP subgroups. Overall, about 45% of the samples were classified into the genetically pure subtypes of DLBCL.

The MCD subtype displayed 82% of cases carrying MYD88L265P or CD79B aberrations (mutation or amplification), with 42% bearing both abnormalities. The MCD subtype showed a frequent gain or amplification of SPIC, encoding a transcription factor that, with IRF4, defines the ABC phenotype and promotes plasmacytic differentiation. Known tumor suppressors in MCD include CDKN2A and CDKN2C, or RPS6KB1 and PTEN. Activating mutations/alterations targeting the NF-kB pathway appeared prominent in MCD genomes, with 76% acquiring a mutation or deletion of HLA-A, HLA-B, or HLA-C and 30% acquiring truncating mutations targeting CD58.

BN2 was dominated by NOTCH pathway aberrations, with 73% acquiring a NOTCH2 mutation or amplification, SPEN mutation, or mutation in DTX1, a NOTCH target gene. BCL6 fusion, the other BN2 hallmark, occurred in 73% of cases. BCL6 fusions were enriched in cases with NOTCH2, SPEN, or DTX1 lesions to a significantly greater extent in BN2 than in non-BN2 cases. Genetic aberrations (mutations or amplifications) targeting regulators of the NF-kB pathway were a prominent feature of BN2. These more often affected TNFAIP3, PRKCB, and BCL10. Other likely gain-of-function events included mutations targeting cyclin D3 and CXCR5, whereas inactivating lesions targeting the immune regulator CD70 suggested immune escape.

N1 was characterized by NOTCH1 mutations and aberrations targeting transcriptional regulators of B-cell differentiation (IRF4, ID3, and BCOR), which may contribute to its plasmacytic phenotype. TNFAIP3 mutations in N1 could reinforce this phenotype by fostering NF-kB-induced IRF4 expression.

EZH2 was enriched for most of the genetic events previously ascribed to GCB-DLBCL, including BCL2 translocation, EZH2 mutation, and REL amplification, as well as inactivation of the tumor suppressors TNFRSF14, CREBBP, EP300, and KMT2D. The germinal-center homing pathway involving S1PR2 and GNA1314 was disrupted in 38% of EZB cases. JAK-STAT signaling was promoted in about half cases by a STAT6 mutation or amplification or by a mutation or deletion targeting SOCS1. PI3K target of rapamycin signaling turned out to be activated in 23% of cases by MTOR mutations or the amplification of MIR17HG. Immune editing was of interest in EZB genomes since 39% acquired lesions in the major histocompatibility complex class II pathway genes CIITA and HLA-DMA.

The four subtypes differed significantly in PFS and OS, with the BN2 and EZB subtypes having much more favorable outcomes than the MCD and N1 subtypes. The predicted 5-year OS rates for the MCD, N1, BN2, and EZB subtypes were 26%, 36%, 65%, and 68%, respectively. Within ABC DLBCL, patients with MCD had significantly inferior survival
as compared with those with BN2, and patients with either MCD or N1 had significantly inferior survival as compared with patients with ABC tumors that were not genetically classified. Within GCB-DLBCL, there was a trend toward inferior OS among patients with EZB as compared with those with other GCB tumors. The COO subgroups and genetic subtypes independently contributed to survival in a multivariate analysis. Conversely, the IPI score did not vary significantly among the genetic subtypes, but the latter significantly added to IPI. A trend toward increased extranodal involvement (e.g., CNS) was a feature of MCD, which reflected the frequent CD79B and MYD88L265P mutations.

On therapeutic grounds, constitutive BCR signaling activation was most frequent in MCD and least frequent in EZB, but genetic alterations involving the BCR cascade occurred in all genetics subtypes, suggesting that constitutive BCR signaling is a pervasive aspect of DLBCL pathogenesis. BN2 was notably enriched for BCR–NF-kB and IKK regulator aberrations. In addition to NF-kB, survival of DLBCL cells turned out to be promoted by antiapoptotic BCL2 family members, which were targeted by genomic amplification or translocation in 17.4% of cases. As expected, BCL2 mRNA levels were significantly higher in EZB tumors with BCL2 translocations than in other EZB tumors. MCD tumors also had high BCL2 mRNA expression as compared with other cases, a finding due to mechanisms other than translocation or amplification.

4.2. Targeted NGS and Bioinformatic-Based Studies

Lacy et al. [54] applied a 293-gene chip to DNA extracted from FFPE tissue samples by using a Covaris LE220. The authors sequenced a large, unselected cohort consisting of 928 DLBCL patients all treated with R-CHOP and provided with full clinical follow-up. Bernoulli mixture-model clustering was applied, and the resulting subtypes analyzed in relation to their clinical characteristics and outcomes. Five molecular subtypes were resolved, termed MYD88, BCL2, SOCS1/SGK1, TET2/SGK1, and NOTCH2, along with an unclassified group. The subtypes characterized by genetic alterations of BCL2, NOTCH2, and MYD88 recapitulated the above-mentioned studies showing good, intermediate, and poor prognosis, respectively. The SOCS1/SGK1 subtype showed biological overlap with primary mediastinal B-cell lymphoma and conferred excellent prognosis. Although not identified as a distinct cluster, NOTCH1 mutation was associated with poor prognosis. The impact of TP53 mutation varied with genomic subtypes, conferring no effect in the NOTCH2 subtype and poor prognosis in the MYD88 subtype. The results obtained by Lacy et al. are summarized in Table 2, where they are also compared with the subtypes reported by Chapuy et al. [51], and Schmitz et al. [52].

Ennishi et al. [55] performed an integrative genomic and transcriptomic analysis of DLBCL using a British Columbia population-based registry. They uncovered recurrent biallelic TMEM30A loss-of-function mutations which were associated with a favorable outcome and were uniquely observed in DLBCL. Using TMEM30A-knockout systems, increased accumulation of chemotherapy drugs was observed in TMEM30A-knockout cell lines and TMEM30A-mutated primary cells, accounting for the improved treatment outcome. Furthermore, they found increased tumor-associated macrophages and an enhanced effect of anti-CD47 blockade limiting tumor growth in TMEM30A-knockout models. By contrast, Ennishi et al. showed that TMEM30A loss-of-function increased B-cell signaling following antigen stimulation—a mechanism conferring selective advantage during B-cell lymphoma development. These findings suggested intrinsic and extrinsic vulnerabilities of cancer cells that can be therapeutically exploited.

Finally, Wright et al. [56] developed an algorithm to determine the probability of a patient’s lymphoma belonging to one of seven genetic subtypes based on its genetic features. This represented a probabilistic classification tool (LymphGen) using any combination of mutational, copy number, and BCL2/BCL6 rearrangement data. Schmitz’s cohort was used as training set, while those from Chapuy et al. and Ennishi et al. were used for validation. Wright et al. developed a model that is summarized in Table 3, which also includes information on potential therapeutic targets related to the genetic subtype of DLBCL.
Table 2. Molecular subtypes of DLBCL according to Lacy et al. in comparison with those of Chapuy et al. and Schmitz et al.

|        | Lacy et al. | Chapuy et al. | Schmitz et al. | Notes                                                                                                                                 |
|--------|-------------|---------------|----------------|---------------------------------------------------------------------------------------------------------------------------------------|
| MYD88  | C5          | MCD           | MYD88          | Strongly associated with ABC. The most robust group in all reports. Contains the most primary PCNSL and testicular lymphoma. Poor prognosis. |
|        |             |               | CD79B          |                                                                                                                                       |
|        |             |               | PIM1           |                                                                                                                                       |
|        |             |               | ETV6           |                                                                                                                                       |
|        |             |               | CDKN2A         |                                                                                                                                       |
|        |             |               | TBL1XK1        |                                                                                                                                       |
| BCL2   | C3          | EZB           | EZH2           | Strongly associated with GCB. Contains most transformed FLs and cases with a concurrent FL. Generally favorable prognosis, although enriched for cases of double-hit lymphoma and MHG. |
|        |             |               | BCL2           |                                                                                                                                       |
|        |             |               | BCL2 translocation |                                                                                                      |
|        |             |               | KMT2D          |                                                                                                                                       |
|        |             |               | TNFRSF14       |                                                                                                                                       |
|        |             |               | CREBBP         |                                                                                                                                       |
|        |             |               | CREBBP2        |                                                                                                                                       |
| SOCS1/ | C4          |               | CD83           | Predominantly GCB. Shares genetic and gene expression features of PMBCL. Associated with the most favorable prognosis.                   |
| SGK1   |             |               | HIST1H1E       |                                                                                                                                       |
|        |             |               | SGK1           |                                                                                                                                       |
|        |             |               | NFKBIA         |                                                                                                                                       |
|        |             |               | NFKBIE         |                                                                                                                                       |
|        |             |               | SOCS1          |                                                                                                                                       |
|        |             |               | BRAF           |                                                                                                                                       |
| TET2/  |              |               | TET2           | A less strongly identifiable subtype. Has very strong similarity to SOCS1/SGK1 but differs by the addition of TET2 and BRAF and the lack of SOCS1 and CD83. Favorable prognosis. |
| SGK1   |              |               | BRAF           |                                                                                                                                       |
|        |              |               | SGK1           |                                                                                                                                       |
|        |              |               | KLHL6          |                                                                                                                                       |
|        |              |               | ID3            |                                                                                                                                       |
| NOTCH2 | C1          | BN2           | BCL10          | Not associated with any COO. Shares mutational similarity to MZI but not enriched for transformed MZLs. Less strongly defined than other subgroups. |
|        |             |               | TNFAIP3        |                                                                                                                                       |
|        |             |               | NOTCH2         |                                                                                                                                       |
|        |             |               | BCL6 translocation |                                                                                                      |
|        |             |               | CCND3          |                                                                                                                                       |
|        |             |               | SPEN           |                                                                                                                                       |
|        |             |               | UBE2A          |                                                                                                                                       |
|        |             |               | CD70           |                                                                                                                                       |
|        |             |               | NOTCH1         | A default category, containing cases that could not be classified elsewhere and no detected mutation. Likely to also contain cases belonging to both NOTCH1 and TP53/CNA subgroups. |
|        |             |               | REL amplification |                                                                                                      |
|        |             |               | TP53           |                                                                                                                                       |
|        |             |               | Frequent deletions | Characterized by TP53 mutation and widespread copy number changes.                                                                 |
### Table 2. Molecular subtypes of DLBCL according to Lacy et al. in 2021, 10, x

| Lacy et al. | Chapuy et al. | Schmitz et al. | Notes |
|-------------|---------------|---------------|-------|
|              | GCB           |              | No detected abnormalities |
|              | N1            |              | Characterized by NOTCH1 mutation, this was significantly elevated in Lacy’s NEC group but only mutated in 2.5% of samples. Associated with poor outcome. |

Cases with no detectable mutation were allocated to the NEC group.

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**Table 3. Implications of genetic subtypes of DLBCL for therapy (from Wright et al., modified).**

| Genotypes | GEP Signature | Related LNH | Targets | 5y-OS |
|-----------|---------------|-------------|---------|-------|
| UNC       | My-T-BCR dependent NF-kB; Immune evasion-MHC | Primary extranodal DLBCL | BCR-dep. NF-kB | 40% All |
| ABC       | B-cell activation | mTORC1 | 37% ABC |
|           | expression; Altered B-cell differentiation; G1-S cell cycle/p53 checkpoint; BCR: IgM>IgG; IgH 4-34" | Transformed WM | BCL2-BCLXe- | |
|           | MYC            | MCL1 | IRAK4 | |
|           | Proliferation | JAK1 | IRF4 | |
|           | BCL2-MCL1 | EZB-MYC+ | |
| ABC       | NOTCH1 signaling | NOTCH1 mutated CLL | NOTCH1 | 27% All |
|           | Altered B cell differentiation | Quiescence | Immune | |
|           | BCR: IgM>IgG | Plasma cells | checkpoints | |
|           | T cell-Myeloid-FDC | |
|           | TP53 inactivation—DNA Damage | p53 | Immune low | 63% All |
|           | Aneuploidy | - | BCR-dep. NF-kB | 33% ABC |
|           | Immune evasion—B2M loss BCR: IgM>IgG; IgH 4-34" | | 100% GCB |
|           | NOTCH2 signaling | B-cell activation | MZL | 67% All |
|           | Altered B cell differentiation | NF-kB | mTORC1 | 76% ABC |
|           | BCR- dependent BCR- | NOTCH | BCL2 | 100% GCB |
|           | evasion—CD70 loss | Proliferation—Cyclin D3 | NOTCH2 | 38% UC |
|           | BCR: IgM>IgG; IgH 4-34" | |
|           | JAK/STAT3 signaling | GC B cell | NPLHL | 84% All |
|           | NF-kB activation | PI3K signaling | PI3K | 81% GCB |
|           | P2RY8-GNA13 activation | Jak2 signaling | JAK2 | |
|           | PI3K signaling | Glycosylation | THRLBCL | |
|           | BCR: IgM>IgG | Stromal | |
|           | myeloma | | |
|           | Chromatin modification | GC LZ (MYC) | FL | 48% MYC^ |
|           | Anti-apoptosis | GC IZ (MYC) | Transformed FL | |
|           | PI3K signaling | BCL6 (MYC) | mTORC1 | 82% MYC^ |
|           | S1PR2-GNA13 inactivation | TCF3 (both) | BL (EZB-MYC) | |
|           | Altered Tfh interactions | Tfh cells (MYC) | EZH2 | |
|           | MYC (EZ-MYC) | Stromal (MYC) | BCL2-MCL1 | |
|           | BCR: IgG>IgM | Immune low (MYC) | |

PCNSL, primary central nervous system lymphoma; FL, follicular lymphoma; MHG, molecular high grade; MZL, marginal zone lymphoma; NLPHL, nodular lymphocyte-predominant Hodgkin lymphoma; THRLBCL, T cell histioyte-.
5. Open Issues and Perspectives

The paper of Lacy et al. [54] was accompanied by a commentary from Morin and Scott [57], who concluded that comprehensive sequencing of a larger number of tumors with the combination of whole-genome and transcriptome sequencing is warranted to develop a new molecular taxonomy which may be concretely translated into clinical benefits. In fact, between 7.5% and 55% of the cases reported by Chapuy et al., Schmitz et al., and Lacy et al. did not fit into any of the major genetic categories they identified [51,53,58].

The fact that the genomic studies hitherto reported show a certain variability in terms of results may depend on different factors, such as the size of the analyzed cohort or heterogeneity of the techniques used (e.g., FF vs. FFPE tissue, whole exome vs. targeted sequencing, and the statistical approach applied), but also on the actual heterogeneity of the lesions occurring in these tumors. For instance, divergent evolution within the same biopsy, which corresponded to different morphologic, phenotypic, and COO features [59], has been reported. Although the distinct components had a common clonal origin and shared the bulk of genetic aberrations, each revealed private mutations, in keeping with the above-mentioned morpho-phenotypic and molecular differences.

The heterogeneity of genetic lesions is much greater than was thought until a couple of years ago. This has been highlighted by liquid biopsy (LB) [59–61]. By ultradep sequencing of the cell-free circulating tumoral DNA (cfDNA) released by neoplastic cells undergoing apoptosis, it has been shown that the global mutational landscape of DLBCL is indeed wider than that observed in diagnostic biopsies, which means that different mutations can occur at different anatomic sites. Once a standardized methodology is developed and the cost per test is reduced, LB can represent a real-time noninvasive tool for disease monitoring.

In fact, patients achieving early molecular response (a 2-log decrease of ctDNA after one cycle of standard chemoimmunotherapy) and major molecular response (a 2.5-log decrease after two cycles) show a significantly superior outcome at 24 months independently of IPI and interim positron emission tomography. Conversely, among treatment-resistant subjects, new mutations are acquired in cfDNA, marking resistant clones selected during the clonal evolution.

The continuous development of sequencing and bioinformatic techniques will allow us to achieve the long searched-for goal of using customized therapies based on the molecular characteristics of each individual tumor. Some approaches do appear to be more easily and cheaply applicable to daily life. Nevertheless, the more comprehensive the bioinformatic approach is, the higher the likelihood of overcoming today’s standard chemoimmunotherapy and designing chemotherapy-free protocols capable of curing most (if not all) patients, with minimal or no toxic effects.

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