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Prognostic molecular biomarkers in diffuse large B-cell lymphoma in the rituximab era and their therapeutic implications

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***Abstract*:** Diffuse large B-cell lymphoma (DLBCL) represents a group of tumors characterized by substantial heterogeneity in terms of their pathological and biological features, a causal factor of their varied clinical outcome. This variation has persisted despite the implementation of rituximab in treatment regimens over the last 20 years. In this context, prognostic biomarkers are of great importance in order to identify high-risk patients that might benefit from treatment intensification or the introduction of novel therapeutic agents. Herein, we review current knowledge on specific immunohistochemical or genetic biomarkers that might be useful in clinical practice. Gene-expression profiling is a tool of special consideration in this effort, as it has enriched our understanding of DLBCL biology and has allowed for the classification of DLBCL by cell-of-origin as well as by more elaborate molecular signatures based on distinct gene-expression profiles. These subgroups might outperform individual biomarkers in terms of prognostication; however, their use in clinical practice is still limited. Moreover, the underappreciated role of the tumor microenvironment in DLBCL prognosis is discussed in terms of prognostic gene-expression signatures, as well as in terms of individual biomarkers of prognostic significance. Finally, the efficacy of novel therapeutic agents for the treatment of DLBCL patients are discussed and an evidence-based therapeutic approach by specific genetic subgroup is suggested.

Keywords: ABC, biomarkers, COO, DLBCL, double-expressor, double-hit, GCB, GEP, prognosis, tumor microenvironment

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Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common lymphoid neoplasm, accounting for ~30% of all non-Hodgkin lymphomas (NHLs). DLBCL is not a single entity; rather, it represents a heterogeneous group of disorders with distinct clinical, pathological, and biological features. The broadest category is termed DLBCL-not otherwise specified (DLBCL, NOS). By definition, these patients do not have specific clinical or pathological characteristics, but they can be further divided into several morphological, molecular, and immunohistochemical subgroups.[1] The addition of rituximab to standard chemotherapy, namely cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP), has undoubtedly improved the outcomes of all DLBCL patients and has been widely accepted as the standard of care. Despite this, a considerable proportion of patients either relapse or experience primary refractory disease and eventually succumb to the disease.[2,3]

The International Prognostic Index (IPI) is currently the most robust prognostic tool for patients with DLBCL. The IPI was introduced and validated in the pre-rituximab era.[4] Although the IPI's prognostic value has been re-assessed in the rituximab era and has been deemed trustworthy, it fails to identify patients with less than 50% of 3-year event-free survival (EFS) who could

potentially benefit from other treatment modalities instead of R-CHOP.[5,6]

Even among patients within the same IPI risk group there is a high variability of outcome. This may reflect the marked genetic and molecular heterogeneity that underlies disease aggressiveness. Therefore, many studies have focused on the identification of biomarkers that may contribute to this phenomenon. Several individual prognostic biomarkers had already been described before the introduction of rituximab; albeit, these are incapable of capturing the great complexity of the underlying biological processes. In the early 2000s, gene expression profiling (GEP) represented an important step towards the elucidation of DLBCL biology and heterogeneity, further optimizing its prognostic stratification.[7–9] GEP studies have identified different molecular DLBCL subtypes related to the cell of origin (COO) as well as several gene expression signatures related to the tumor microenvironment (TME). Both are of prognostic significance. In addition, GEP studies have highlighted the prognostic value of many genes and have led to the discovery of several molecular pathways that may serve as therapeutic targets.

The addition of rituximab to the CHOP regimen has altered the significance of certain established prognostic factors, either as a result of statistical reasons (the marked improvement in outcome of patients with DLBCL leads to fewer events) or directly through its mechanism of action. Therefore, previously well-described prognostic biomarkers have been re-evaluated in the rituximab era. The emergence of novel agents for the treatment of DLBCL patients highlights the need for the establishment of their prognostic relevance for patients treated with these therapeutic modalities.

The present review summarizes the current knowledge regarding biological prognostic factors in DLBCL-NOS in the rituximab era. In addition, it provides insights into the efficacy of novel agents in the frontline therapy of high-risk DLBCL patients.

Genetic subgroups of prognostic significance

COO

GEP assessed by DNA microarray allows for the simultaneous profiling of the expression of thousands of genes in cells while obtaining a detailed record of their expression. Alizadeh *et al.* identified two distinct molecular subgroups of DLBCL with gene expression patterns indicative of different stages of B-cell differentiation, as well as highly distinct overall survival (OS). The first subgroup was composed of DLBCL with a gene expression signature resembling that of germinal center B-cells (Germinal Center B-like, GCB), whereas the second contained DLBCL with expression of genes which are induced during *in vitro* activation of peripheral blood B-cells (Activated B-cell, ABC).[7] Rosenwald *et al.* demonstrated the presence of a third molecular subgroup, called type 3 or unclassified DLBLC, that included cases not expressing either set of genes characteristic of GCB or ABC subgroups. The distribution of cases among these different subgroups was 47.9%, 30.4%, and 21%, for GCB, ABC, and type 3 DLBCL, respectively. 5-year OS rates were significantly higher for the GCB DLBCL patients compared with the other subgroups independent of their IPI. Furthermore, four distinct gene-expression signatures [GCB, proliferation, major histocompatibility complex (MHC) class II, and lymph node] with prognostic significance were identified.[8] The prognostic value of DLBCL subtyping by GEP analysis has been re-evaluated in the rituximab era. Lenz *et al.*[9] reported that GCB DLBCL patients had significantly higher OS and progression-free survival (PFS) than ABC DLBCL patients, a finding highly consistent in several studies.[10,11]

Based on the above studies, the molecular classification of DLBCL by COO has been recognized as the gold-standard approach for the molecular classification of DLBCL and provides valuable prognostic information independently of the IPI. However, these techniques are not available for routine use and require fresh or frozen tissue samples with adequate amounts of RNA. To overcome these limitations, many researchers have tried to determine COO by applying GEP techniques to formalin-fixed paraffin-embedded tissues (FFPET) with high accuracy.[12–19] Among the suggested approaches, Lymph2Cx, a digital GEP assay based on a panel of 20 genes, has been validated and demonstrated its non-inferiority to GEP determination of COO.[16]

The unquestionable prognostic significance of COO in DLBCL led many researchers to develop

prediction models based on simpler techniques such as immunohistochemistry (IHC) in FFPET. The IHC algorithms uses antibodies specific to GCB and ABC-markers. It assesses protein expression in order to classify DLBCL cases as either GCB or non-GCB. The most widely utilized of them, Hans algorithm, uses *CD10*, *BCL6* and *MUM1*.[20] Although in accordance with GEP, its prognostic significance in the rituximab era has been disputed. A recent meta-analysis showed that COO determined by the Hans algorithm was predictive of PFS but not OS in patients treated with rituximab.[21] A recent study by Adulla *et al.* in 359 DLBCL patients confirmed the inferiority of the Hans algorithm to predict OS.[22] Most strikingly, a recent study by Cho *et al.*[23] failed to demonstrate any prognostic effect of the Hans algorithm in PFS or OS; in contrast, COO determination by Lymph2Cx was strongly predictive of both outcomes. The Choi algorithm, developed in the rituximab era, utilizes *CD10, MUM1, GCET, FOXP1* and *BCL6*, allowing for a more accurate COO designation;[24] however, similar to the Hans algorithm, its prognostic significance might be limited.[21] Several other algorithms (Colomo, modified Hans, modified Choi, Visco–Young, Tally, Muris, Natkuman, and Nyman) have been proposed;[25–30] albeit, results regarding the prognostic significance of these algorithms remain equivocal.[31]

The poor prognostic performance of the IHC algorithms could be attributed to their inherently binary nature, as they classify cases as GCB or non-GCB. Therefore, cases unclassified by GEP (type 3) are inevitably misclassified by the algorithms, hindering their prognostic value. Moreover, as shown by the Lunenburg Lymphoma Biomarker Consortium Study (LLBC), these results could be explained by sampling techniques and technical issues, as well as by inter-observer variation.[32] The optimization of staining techniques and scoring criteria has failed to improve the prognostic value of IHC algorithms.[33] To summarize, the IHC algorithms remain suboptimal for a prognostically relevant classification of DLBCL, and GEP represents the gold-standard for COO classification. Of note, novel assays such as Lymph2Cx are applicable in FFPET, overcoming limitations of earlier GEP assays. Other novel FFPET-based approaches utilize multiplex quantitative real-time polymerase chain reaction (qRT-PCR) and next-generation sequencing (NGS) in order to target a specific panel of genes. These approaches are highly accordant to GEP and highly predictive of PFS and OS.[34,35]

Gene-expression models

Findings from GEP analysis drove researchers to pursue prognostic models that incorporate the expression of several genes. Lossos *et al.*[36] evaluated a qRT-PCR model based on the expression of six genes (*LMO2, BCL6, FN1, CCND2, SCYA3*, and *BCL2*) that is also applicable in FFPET. In the rituximab era, the model has been shown to predict OS but not EFS.[37,38] Another model incorporating four genes of the COO signature (*LMO2*, *MME*, *LPP*, and *FOXP1*) and two immune-related genes (*APOBEC3G* and *RAB33A*) has been proposed; however, as it has been based in a small cohort of elderly patients and has not been externally validated, no conclusions regarding its prognostic significance can be drawn.[11] In a more simplified approach, Alizadeh *et al.* created a two-gene model based on the expression of *LMO2* and a TME-related gene (*TNFRSF9*) in FFPET. The two-gene model was an independent predictor of OS, independent of COO and IPI. A composite score integrating these gene-expression with IPI could stratify patients in low-, intermediate- and high-risk groups with distinct PFS and OS.[39] More recently, Green *et al.* proposed a model incorporating the expression of *LMO2* and *HLADQA1* as well as three gene interactions for *GCSAMxMIB1*, *GCSAMxCTGF*, and *FOXP1xPDE4B* that predicted PFS and OS independently of IPI. As the complexity of this model might hinder its applicability, a simplified version has been proposed, comprising *LMO2*, *BCL2* expression, and IPI. This showed comparable performance to the more complex model and was validated in an independent cohort.[40]

Apart from qRT-PCR, other gene-expression assays have been evaluated for prognostication in DLBCL. Among them, quantitative S1 nuclease protection assay (qNPA) in FFPET has been used to assess the expression of several genes. In this context, Rimsza *et al.*[12] demonstrated that a model comprising *HLA-DRB* and *MYC* expression assessed by qNPA could predict OS and PFS.

In conclusion, gene-expression assays applicable in FFPET have allowed for the development of

prognostic models incorporating gene-expression information as well as clinical factors. However, lacking external validation, the results of these studies should be interpreted cautiously. Moreover, no consensus on the optimal combination of genes for the prediction of the clinical outcome as well as the methodology for gene-expression assessment have been reached. This has largely hindered the reproducibility of results. Of interest among the investigated genes, *LMO2* has been consistently associated with favorable outcome; however, further studies are needed to elucidate the appropriate gene combination that would comprise a widely accepted prognostic model.

Novel molecular subgroups

Recent reports have highlighted the presence of residual heterogeneity in DLBCL prognosis, even among the well-characterized COO subgroups. Several studies have tried to refine the molecular classification of DLBCL in this context. Reddy *et al.* integrated whole exome sequencing and transcriptome sequencing to identify 150 driver genes in 1001 DLBCL patients. Their mutational and gene-expression profiles were used to construct a prognostic model that outperformed other established prognostic approaches such as COO determination and IPI. According to this model, 39 subgroups emerged, with significant discrepancies in OS. The subgroup with the most dismal prognosis comprised cases with *MYC* genetic and/or gene-expression aberrations irrespective of COO. In contrast, GCB-DLBCL with *CD70* alterations represented the subgroup with the most favorable outcome.[41]

In another approach, Schmitz *et al.* utilized whole-exome and transcriptome sequencing, array-based DNA copy-number analysis, and targeted resequencing of 372 genes in 574 DLBCL cases. They managed to classify 44.8% of cases into four distinct subgroups: MCD (combined *MYD88*^{L265P} and *CD79B* mutations), BN2 (*BCL6* fusions and *NOTCH2* mutations), N1 (*NOTCH1* mutations), and EZB (*EZH2* and *BCL2* rearrangements). The MCD and N1 subtypes where mostly composed of ABC-DLBCL, EZB composed mostly of GCB-DLBCL, whereas BN2 was equally prevalent in all COO groups. The four subtypes had statistically significant differences in PFS and OS; the 5-year OS for the MCD, N1, BN2, and EZB subtypes were 26%, 36%, 65%, and 68%, respectively. Within the ABC subgroup, BN2 represented the subtype with the most favorable OS and PFS, whereas N1 and MCD had dismal prognosis compared with ABC-NOS and BN2; within the GCB subtype, EZB subtype demonstrated inferior survival compared with GCB-NOS. Notably, MCD and BN2 demonstrated recurrent B-cell receptor (BCR)-dependent *NF-κB* activation, and N1 revealed a T-cell gene-expression signature with potential therapeutic implications.[42]

Most recently, Chapuy *et al.* analyzed 304 DLBCL samples for recurrent low-frequency alterations, mutations, somatic copy number alterations (SCNAs), and structural variants (SVs). They identified five DLBCL subsets (C1-C5) with distinct clinical behavior. Within the ABC group, C1 (*BCL6* SVs and *NOTCH2* mutations) represents a subgroup with favorable prognosis, whereas C5 (gains in *BCL2* and/or mutations in *MYD88*^{L265P}, *CD79B*, *ETV6*, *PIM1*, *GRHPR, TBL1XR1*, and *BTG1*) showed inferior outcome. On the other hand, two subgroups were identified within the GCB group, those being C3 (*BCL2* mutations and SVs along with mutations in epigenetic modifiers, *KMT2D*, *CREBBP*, and *EZH2)* which was characterized by inferior outcome, and C4 with favorable prognosis characterized by aberrations in BCR/PI3K signaling, *NF-κB* and *RAS/JAK/STAT* pathway (mutations in *CD83*, *CD58*, and *CD70*, *RHOA*, *GNA13*, and *SGK1*, *CARD11*, *NFKBIE*, and *NFKBIA*, and *BRAF*, *STAT3*).

The remaining cluster, named C2, is composed of COO-independent DLBCL with biallelic inactivation of *TP53* as well as copy loss of *CDKN2A*, and *RB1*. It demonstrated an intermediate OS between C1, C4 and C3, C5.[43] It should be noted that the C1, C3, and C5 groups partially overlap with the BN2, EZB, and MCD groups outlined by Schmitz *et al*.[42]

Lacy *et al.* proposed a similar classification scheme based on the targeted sequencing of 928 DLBCL FFPET samples. Five distinct subsets were identified (MYD88, BCL2, SOCS1/SGK1, TET2/SGK1, and NOTCH2), which significantly overlap with those described by Schmitz *et al*.[42] and Chapuy *et al*.[43] Indeed, MYD88 overlaps with MCD and C5, BCL2 with EZB and C3, and NOTCH2 with BN2 and C1. Regarding SOCS1/SGK1 and TET2/SGK1, overlap is seen with the

C4 subgroup; however, they might represent distinct subgroups, based on the augmented expression of *TET2* and *BRAF* in the latter. Moreover, although both subgroups have relatively good prognosis, the former group is associated with a more favorable outcome.[44]

Almost concurrently, Wright *et al.* proposed a refinement of the classification scheme by Schmitz *et al.*,[42] aiming to eliminate the previously unclassifiable cases. In this context, they proposed two additional subgroups named A53 and ST2. The former aligns with the C2 subgroup by Chapuy *et al.*,[43] as it is composed of cases enriched for *TP53* mutations, whereas the ST2 aligns with the TET2/SGK1 subgroup which was described previously. Similarly, to the previous classification schemes, significant differences in clinical outcome were noted among different subgroups.[45]

A considerable portion of DLBCL remains unclassified, even by the implementation of the novel approaches discussed so far; as a result, prognostic ability is hampered. Alkodsi *et al.* proposed a classification scheme which is based on the somatic hypermutation (SHM) patterns of 36 target genes. They managed to identify four distinct subgroups named SHM1-4 that allowed prognostic stratification of patients within the ABC and GCB subtypes, but also within unclassified DLBCL. In this scheme, ABC is subdivided into SHM2 with aberrant activation of the BCR signaling pathway and the worse outcome among all SHM subgroups, while SHM4 is characterized by *BCL6* fusions as well as *CD70* and *BCL10* mutations. On the other hand, GCB group is subdivided into SHM1 with high frequency of *BCL2* and *MYC* aberrations in addition to mutation of chromatin modifying genes, showing poor outcome with conventional immunochemotherapy, and SHM3 exhibits aberrant JAK/STAT signaling and the most favorable outcome compared with the other subgroups.[46]

The inter-correlation of the novel genetic subgroups is depicted in Figure 1. Key genetic features of each subgroup are summarized in Tables 1 and 2. To summarize, genomic studies have disentangled the complex genomic infrastructure of DLBCL, allowing for the subclassification of cases in prognostically relevant subgroups with shared genetic aberrations. Although most of the techniques used in the described studies might be time-consuming and excessively expensive to be applied in clinical practice, targeted NGS, which in addition, is applicable in FFPET, might represent an appealing approach for genetic classification in general practice. For this to occur, validation in prospective studies is needed. Nonetheless, classification in well-characterized genetic subgroups might provide the basis for designing of meaningful preclinical and clinical studies.

The tumor microenvironment

Although the role of the TME has been widely established in other lymphoid malignancies such as Hodgkin lymphoma, its role in DLBCL remains controversial. In 2008 Lenz *et al.* identified two gene-expression signatures, stromal-1 and stromal-2, which reflected discrepant composition of TME in DLBCL. The favorable stromal-1 signature, associated with a phenotype characterized by abundant extracellular matrix and infiltration by histocytes, was enriched for genes encoding for the major components of the extracellular matrix and the anti-angiogenic factor thrombospondin, along with modifiers of collagen synthesis and proteins implicated in the remodeling of extracellular matrix. In contrast, the less favorable signature stromal-2 was mainly enriched for genes encoding for markers of endothelial cells and regulators of angiogenesis, and it was characterized by high blood-vessel density.[9] However, the lack of reproducible methodology in FFPET hampered its applicability, despite the clear prognostic implications of TME. Nonetheless, vascular endothelial growth factor receptor 2 (VEGFR2) expression and high microvessel density, assessed by IHC, correlate with poor outcome,[47,48] as opposed to expression of VEGFR1, which has been associated with a more favorable prognosis.[48] Notably, IHC expression of *HIF1a* might confer improved prognosis in DLBCL, despite promoting angiogenesis, through upregulation of several genes within the favorable stromal-1 signature.[49] As expected, IHC expression of SPARC, overexpressed within the favorable stromal-1 signature, has been associated with improved OS and PFS independently of IPI; however, its prognostic effect is restricted within the ABC subgroup.[50] An IHC-based predictive model incorporating the non-GCB subtype, low expression of SPARC (<5%), and high microvessel density has been suggested.[51]

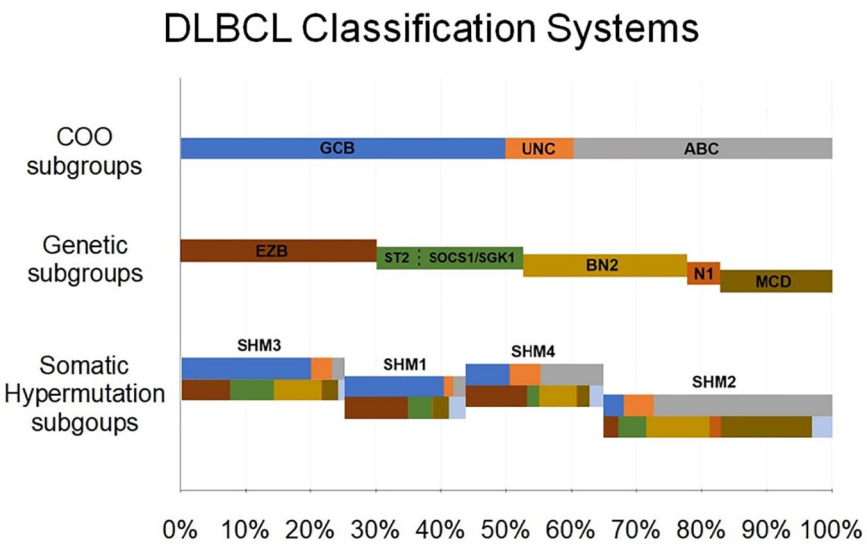

Figure 1. Schematic representation of the relationship between cell-of-origin (COO), genetic (Wright *et al.*[45]), and somatic hypermutation (Alkodsi *et al.*[46]) subgroups in diffuse large B-cell lymphoma (DLBCL). The upper panel depicts the relative proportion of germinal center-like B cells (GCBs), activated B cell (ABC), and unclassified, among all DLBCL cases. The intermediate panel depicts the molecular subgroups, identified by gene-expression profiling; their relative position to the upper panel correlates to the association of these subgroups with COO (the A53 subgroup is not depicted on this Figure as there is no correlation with COO). The lower panel depicts the somatic hypermutation subgroups; the chromatic code of the upper part of each bar depicts the correlation of each subgroup to COO, whereas the chromatic code of the lower part depicts correlation to molecular subgroups. The relative width of each bar corresponds to the relative proportion of each subgroup among all DLBCL cases.

Table 1. Key genetic features and 5-year overall survival by molecular diffuse large B-cell lymphoma (DLBCL) subgroups, identified by gene-expression profiling, by Schmitz *et al.*,[42] Chapuy *et al.*,[43] Lacy *et al.*,[44] and Wright *et al.*[45].

Molecular subgroups				Key genetic aberrations	5-year overall survival
Schmitz *et al.*[42]	**Chapuy *et al.*[43]**	**Lacy *et al.*[44]**	**Wright *et al.*[45]**		
BN2	C1	NOTCH2	BN2	*BCL6, NOTCH2, TNFAIP3, SPEN, BCL10, TMEM30A*	67%
	C2		A53	*TP53, TP53BP1*	63%
EZB	C3	BCL2	EZB-MYC+	*BCL2, EZH2, KMT2D, TNFRSF14, CREBBP, GNA13, MEF2B, IRF8*	48% (DHITsig-positive)
			EZB-MYC-		82% (DHITsig-negative)
	C4	TET2/SGK1	ST2	*SGK1, TET2, ZFP36L1*	84%
		SOCS1/SGK1		*SOCS1, SGK1, CD38*	80%
MCD	C5	MYD88	MCD	*MYD88, CD79A/B, PIM1, TBL1XR1, PRDM1, SPIB, BTG1/2, CDKN2A*	40%
N1			N1	*NOTCH1, ID3, KLHL6*	27%

Several studies have assessed the prognostic role of immune composition of TME in DLBCL. Ciavarella *et al.* demonstrated that higher proportions of myofibroblasts, dendritic cells, and CD4+ T cells correlated with superior OS, whereas activated natural killer (NK) and plasma

cells (PCs) correlated with inferior outcome. TME gene-expression profiling identified three clusters (low, intermediate, high-expression) that predicted OS independently of COO. A classification scheme integrating COO and TME subtypes has also been proposed.[52] A high number of FOXP3+ regulatory T-cells have been associated with inferior outcomes in most of these studies.[53,54] Moreover, lymphoma-associated macrophages (LAMs) play a crucial part within the TME; however, distinct subsets of LAMs may occur in opposing modes. M2 macrophages are immunosuppressive and promote tumor evasion, whereas M1 macrophages induce immune response and exert anti-lymphomatic action. Therefore, studies of individual macrophage markers have yielded conflicting results.[55] To overcome this inherent limitation, Stagger *et al.*[56] constructed a LAM interaction signature (LAMIS) that was applied to 466 FFPET samples, demonstrating that high expression of this signature was predictive of inferior PFS and OS, irrespective of IPI and COO.

Most recently, the role of the programmed cell death protein 1 (PD-1)/PD-L(ligand)1 axis has been highlighted as a key mechanism of immune evasion, both in solid tumors and in DLBCL. When PD-L1 is expressed by tumor cells, it interacts with PD-1 in T-cells leading to T-cell anergy and immune evasion.[57] PD-L1 overexpression is observed in ~20% of DLBCL cases due to gains, amplification, or rearrangements affecting the PD-L1 locus.[58] Several studies have shown that the overexpression of PD-L1 by tumor cells correlates with poor OS and PFS, independent of IPI and COO.[59–61] Notably, PD-L1 expression strongly correlates with Epstein–Barr virus infection and the ABC subtype;[61] on the other hand, PD-1 expression by T-cells within the TME might predict a more favorable outcome.[62,63] Other mechanisms of immune evasion include the downregulation of several genes comprising the MHC class II and inactivating mutations of the *B2M* gene, encoding for β2-microglobulin as well as downregulation of *CD58*, which is involved in NK cell responses.[64] An association between these immune evasion mechanisms and OS has been noted.[65–68]

To evaluate the role of different subsets of immune cells in the TME, Keane *et al.* assessed the expression of immune effector and checkpoint genes in 252 FFPET DLBCL. They demonstrated that the expression of immune effectors (T/NK) correlates with the expression of markers associated with macrophages and the PD/PD-L1 axis. Thus, the anti-lymphomatic action exerted by the former cells is truncated. Therefore, the CD4*CD8:M2*PD-L1 ratio, assessed by digital hybridization, was used to stratify patients in two prognostic groups irrespective of IPI and COO. Patients with a high ratio experienced more favorable PFS and OS, as well as better response rates to R-CHOP compared with patients with low ratio.[69]

In a pivotal transcriptomic study of more than 4000 DLBCL samples, Kotlov *et al.* characterized four clusters termed germinal center-like (GC-like), mesenchymal (MS), inflammatory (IN), and depleted (DP). GC-like cluster resembles the cellular composition of normal germinal center, whereas MS is characterized by increased endothelial cells and fibroblasts as well as abundant extracellular matrix. Both clusters, enriched within the GCB subgroup, are associated with favorable PFS and OS. On the other hand, IN cluster, characterized by a highly inflammatory TME rich in neutrophils and macrophages and the DP cluster, showing a deserted TME, are associated by inferior prognosis, irrespective of COO designation. Notably, the TME clusters are distributed across all genetic subgroups, suggesting that a classification system based on the composition of TME may serve an auxiliary role to the genetic classification for the prognostic characterization, and therapeutic management of DLBCL patients.[70] The validation of recent findings in large prospective studies and the development of simplified techniques for application in FFPET is needed for the adoption of TME clustering in clinical

Table 2. Key genetic aberrations of somatic hypermutation subgroups in diffuse large B-cell lymphoma (DLBCL), identified by Alkodsi *et al.*[46].

Subgroup	Key genetic features
SHM1	*EZH2, KMT2D, CREBBP, MYC, BCL2, GNA13, GNA12, P2RY8*, Chr7, Chr8 gains
SHM2	*MYD88, CD79B, CDKN2A, PIM1, MPEG1, ETV6, IRF4*, Chr3, Chr18 gains
SHM3	*SOCS1, STAT3, STAT6, TNFAIP3, SGK1, IRF8*, Chr3, Chr7 Chr18 gains
SHM4	*BCL6, CD70, BCL10, SPEN, MYD88* (not L265P), *HLA-A,B,C*

Chr, Chromosome.

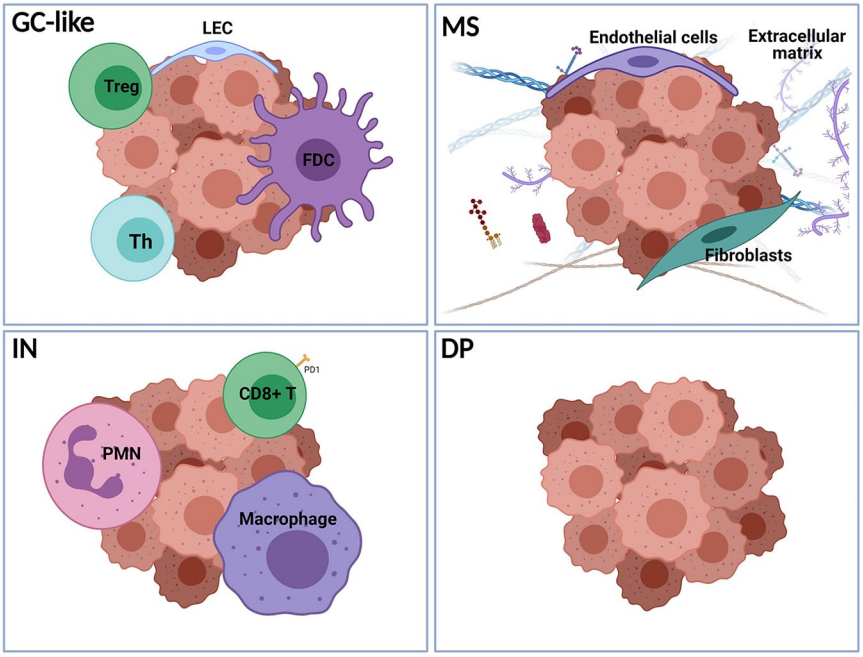

Figure 2. Schematic representation of the predominant cells in the microenvironment of the four DLBCL subgroups with prognostic significance, described by Kotlov *et al.*[70] (GC-like, germinal center-like; MS, mesenchymal; IN, inflammatory; and DP, depleted).
LEC, lymphatic endothelial cells; PMN, polymorphonuclear cells; Th, T-helper cells; Treg, T-regulatory cells. (Created with BioRender.com).

practice. Until then, the considerable prognostic role of TME can be assessed by IHC of specific markers implicated in angiogenesis or immune response as well as targeted sequencing. A graphical representation of the TME clusters based on their cellular composition of TME is depicted in Figure 2.

Double-hit, triple-hit, and double-expressor lymphomas

BCL2 is overexpressed in 47-58% of DLBCL patients.[71] In the GCB subgroup and particularly within the EZB genetic subgroup, *BCL2* upregulation is mainly attributed to the rearrangement t(14;18)(q32;q21).[72] In contrast, the ABC subgroup is characterized by the 18q21 chromosome locus gain/amplification.[73] In the rituximab era, *BCL2* rearrangement remain predictive of significantly inferior OS among patients with the GCB subtype, irrespective of *MYC* status. On the other hand, *BCL2* amplification or gains are predictive of inferior OS and PFS within the ABC subgroup.[74,75] *BCL2* overexpression has retained its prognostic ability solely within the GCB subgroup.[76,77] *MYC* rearrangements can be identified in 5-14% of DBCL patients, and more commonly with the GCB subgroup.[78] The rearrangement t(8;14)(q24;q32) represents the most typical, bridging *MYC* to the immunoglobulin heavy chain gene locus; however, in 53% of these cases, the partner is not an immunoglobulin (IG) gene.[79] Although numerous studies have demonstrated the negative prognostic effect of *MYC* rearrangements on PFS and OS, as well as on central nervous system (CNS) relapse risk, the prognostic significance of isolated *MYC* rearrangements has been disputed.[80–83] It has been shown that cases with isolated *MYC* rearrangements demonstrate an OS and PFS approximating that of non-rearranged cases. This highlights that the detrimental effect of *MYC* rearrangements is highly dependent on a second genetic hit, particularly in *BCL2* or *BCL6* and *TP53*, which are found in up to 80% of cases.[84,85] Similarly, overexpression of the MYC protein, which is demonstrated in ~30% of DLBCL cases, has been considered an independent prognostic factor for OS and PFS irrespective of the underlying mechanism; however, its prognostic effect is modified by concurrent *BCL2* or *BCL6* genetic aberrations or

overexpression of the respective proteins.[85] *BCL6*, located in the 3q27 chromosome, represents a major marker of GCB origin;[20,86] albeit, *BCL6* rearrangements are twice as common within the ABC subgroup[87] and confer a negative effect to OS and PFS as is evident in a recent meta-analysis.[88] On the other hand, BCL6 overexpression, mainly attributed to gene mutations, represents a prominent feature of the GCB subgroup.[87] High *BCL6* mRNA and protein expression have been, and still are, strong predictors of favorable outcome in DLBCL patients.[89,90] It should be noted, however, that the prognostic significance of *BCL6* rearrangements and overexpression might reflect its higher prevalence within the prognostically significant GCB and ABC subgroups, respectively.

In 58–63% of the cases, the *MYC* rearrangement is accompanied by at least one additional rearrangement, most commonly of *BCL2* or *BCL6*. Cases harboring *MYC* and *BCL2* or *BCL6* rearrangements are termed double-hit (DH) lymphomas, whereas concurrent rearrangement of all three genes characterizes the subset of triple-hit (TH) lymphomas.[91] In the 2016 revision of WHO classifications, DH and TH lymphomas with DLBCL morphological features were excluded from the DLBCL-NOS category, and have been assigned to a new diagnostic entity termed high-grade B-cell lymphomas (HGBL) with *MYC* and *BCL2* and/or *BCL6* (HGBL-DH/TH).[1] HGBL-DH/TH accounts for 7.9% of tumors with DLBCL morphology; among them, DH-BCL2 and TH lymphomas represent more than 80% of cases, whereas DH-BCL6 lymphomas are relatively rare, accounting for 18.6% of cases. Most strikingly, DH-BCL2 and TH lymphomas are almost invariably associated with the GCB subgroup, whereas DH-BCL6 is distributed equally among COO subgroups.[92]

DH and TH have been associated with an inferior outcome, predicting an aggressive clinical course and poor response to R-CHOP.[82,85,93,94] As 5-year OS and PFS has been reported to be rather poor (27% and 18%, respectively) in R-CHOP treated patients,[85] more aggressive therapeutic approaches have been suggested; however, several ongoing controversies should be highlighted. First, DH and TH are not invariably associated with overexpression of the respective proteins. Several studies have demonstrated that these cases, which represent a non-negligible proportion of ~20% of DHs, have a more favorable prognostic profile.[85,95,96] Moreover, the prognostic significance of DH-BCL6 cases remains equivocal. Older studies demonstrated that DH-BCL6 is associated with dismal outcomes;[97,98] in contrast, more recent studies have showed that the co-occurrence of *MYC* and *BCL6* re-arrangements is not associated with an inferior outcome in DLBCL.[99,100] Recent findings have also underscored the differential role of the partner gene in *MYC* rearrangement in prognosis among DH and TH DLBCL patients. A recent large study by Rosenwald *et al.*[101] showed that DH and TH cases harboring *MYC* rearrangements to non-immunoglobulin genes showed no significant differences in terms of OS and PFS, compared with non-DH/TH cases. Moreover, cases with gene amplifications rather than rearrangements have been identified, however the prognostic significance of these abnormalities remains controvertial.[102]

Previous limitations have led researchers to utilize GEP to identify DH and TH cases with genuine prognostic significance. Ennishi *et al.* identified a 104-gene DH signature (DHITsig) which characterizes most DH/TH cases. This signature was identified in 27% of cases within the GCB subgroup; among them, only one half were DH/TH by fluorescence *in situ* hybridization (FISH). Most strikingly, it was shown that DHITsig-positive (DHITsig +ve) cases had dismal outcome, accompanied by poor response rates to R-CHOP, irrespective of their *MYC*, *BCL2*, and *BCL6* rearrangement status.[103] Further analysis using whole-genome sequencing identified genetic alterations to *MYC* and *BCL2* which are undetectable by conventional FISH in most of the non-DH/TH DHITsig +ve cases. Notably, six out of 20 analyzed cases harbored rearrangements cryptic to conventional FISH, whereas genetic events affecting both *MYC* and *BCL2* were identified in seven additional cases.[104] Almost concurrently, Sha *et al.* identified a molecular high-grade (MHG) gene expression signature characteristic of DH/TH cases which extends beyond them, within the GCB subgroup. This signature was predictive of inferior outcome irrespective of DH/TH status.[105] The two genetic signatures are highly correlated and characterize tumors originating from the intermediate germinal center zone, particularly enriched within the EZB subgroup. Tumors within the EZB subgroup can be further classified by the presence of

DHIT signature into EZB-MYC+ and EZB-MYC-. EZB-MYC+ might represent highly aggressive tumors arising from a dark zone with a 5-year OS of 48%. In contrast, EZB-MYC- tumors, arising from the light zone, have a more favorable prognosis (5-year OS: 82%).[45] DHITsig +ve DLBCL shows high proliferation and immune evasion, owing to the frequent loss of MHC antigens and their lymphocyte-depleted microenvironment.[103,105] Indeed, the DHITsig +ve subgroup was significantly enriched within the DP TME subgroup by Kotlov *et al.*[70] Notably, stratification by TME composition retains its prognostic significance even in this subgroup.

Lymphomas with a high co-expression of MYC and BCL2 proteins are called double expressor lymphomas (DE) and should not be confused with DH/TH lymphomas as they do not represent a distinct biological subgroup but a prognostically relevant subcategory. MYC and BCL2 protein co-expression by IHC is present in 21%-29% of DLBCL patients and undoubtedly confers poor prognosis (5-year OS: ~40%).[85,106] Furthermore, MYC/BCL2 co-expression was also associated with poor prognosis in another large study of 893 DLBCL patients treated with R-CHOP (5-year OS: 30% *versus* 75%).[107] DEs are more common within the ABC subgroup, particularly when HGBL-DH/TH are excluded, contributing to the inferior prognosis of cases fitting in this subgroup.[80] The underlying mechanism of DE differs among COO subgroups; within GCB, overexpression is attributed to gene re-arrangements, whereas in the ABC subgroup, overexpression represents the sequela of a complex genetic interplay involving gene amplifications and aberrations in B-cell receptor and *NF-kB* signaling.[77] Recently Horn *et al.* proposed a prognostic model based on MYC protein expression and *MYC* rearrangement status in combination with *BCL2* and *BCL6* expression status. *MYC* rearrangements, MYC^{high}, $BCL2^{high}$, and $BCL6^{low}$ protein expression were predictive of inferior survival independently of IPI.[90] Recently, the incorporation of *CD37, MYC*, and *BCL2* to the R-IPI has shown to augment its prognostic power.[108] The distribution of genuine and cryptic DH, DE, and DHITsig +ve cases among GCB and ABC subgroups along with their overlap is depicted in Figure 3.

In conclusion, DE and DH lymphomas seem to predict a more aggressive clinical course, underlying the need for early identification, and potentially treatment intensification as well as the introduction of novel agents; however, limitations of the current IHC and FISH methods hamper the classification in biologically distinct subgroups. In this context, gene-expression signatures might serve for the accurate distinction between these subgroups. Notably, a DHITsig module has been incorporated in the Lymph3Cx assay for COO characterization, allowing for the application in FFPET in clinical practice.

Other biomarkers

TP53 mutations, found in ~20% of DLBCL patients among both COO subgroups, tend to be more common among cases with *MYC* rearrangements. *TP53* mutations correlate with unfavorable disease characteristics and predict inferior OS and PFS independent of IPI and COO.[109–111] In contrast, the prognostic significance of *TP53* deletions and or del(17p) in the absence of a mutated allele remains controversial.[109,112] In regards to IHC, strong TP53 expression (in at least 50% of the malignant cells) might be an independent predictor of shorter OS;[113] however, the absence of a concurrent *TP53* mutation negates the prognostic significance of the respective protein overexpression.[109]

High proliferation rate, reflected by high expression of Ki-67, has been predictive of inferior outcomes in DLBCL, as demonstrated by a recent meta-analysis.[114] In addition, recent research has shown that the prognostic value of Ki-67 might be more pronounced within the non-GCB subgroup.[115,116]

De novo CD5+ DLBCL, accounting for 5–22% of DLBCL cases, represents a distinct immunohistochemical subgroup within DLBCL-NOS.[117] Most commonly of ABC origin (82%), this subgroup highly correlates with double *MYC*/*BCL2* overexpression.[118] CD5+ cases tend to present with more advanced disease, whereas CNS recurrence is particularly high (13% *versus* 5% for CD5- DLBCL).[119] Despite the introduction of rituximab, the prognosis for CD5+ DLBCL remains dismal, with 5-year OS and PFS rates of 35.5% and 29.6% respectively, and high CNS relapse rates.[120–122] The aggressiveness of CD5+ DLBCL has been attributed to several mechanisms, including the inhibition of BCR signaling as well as the overexpression of IL-10, BCL2, cyclin D2, and CXCR4.[117]

Patients with reduced CD20 expression and high CD19 expression (discordant CD20), identified through flow cytometry (FCM), have been shown to have inferior OS independently of their IPI.[123,124] Notably, IHC assessment might not be a reliable method for estimation of CD20 expression level compared with FCM; albeit, the latter requires fresh tissue samples. To overcome the inherent limitation of FCM, a semi-quantitative IHC method has been developed for the assessment of CD20 expression in FFPET, verifying the prognostic significance of low CD20 expression.[125]

CD30 was overexpressed in 14% of patients and was correlated with superior 5-year OS and PFS independent of COO and IPI. CD30+ DLBCL demonstrated a distinct GEP signature, characterized by the downregulation of *NF-κB* and BCR pathways, potentially explaining the favorable profile of this DLBCL subset. Interestingly, a strong correlation between CD30 expression and EBV infection has been observed. As a side note, EBER seems to negate the favorable effect of CD30, as cases co-expressing EBER and CD30 had a dismal outcome.[126]

With regards to molecules implicated in apoptosis, the role of *BCL2* has been thoroughly assessed previously, in contrast to other genes that have not been evaluated as much. Recently, it has been shown that high *BCL2L12* expression, assessed both at the mRNA level and via IHC, confers a more favorable outcome in patients with DLBCL, irrespective of COO and IPI.[127] Expression of other anti-apoptotic genes such as *BIRC5* (survivin) and *XIAP* has been reported to confer an adverse effect on prognosis,[128,129] while results regarding *CFLAR* (c-FLIP) are contradictory.[130,131] On the other hand, the expression of *CASP3* and *CDKN1A*, a downstream effector of *TP53*, may correlate with favorable outcome.[132,133] Markovic *et al.*[134] have created an apoptotic score based on the IHC expression of *CASP3*, *CD95*, *CFLAR*, *BIRC5*, *XIAP*, and *BCL2* that predicts OS, whereas Pasanen *et al.*[135] have designed a prognostic score among GCB DLBCL patients which is based on the cell cycle-regulating proteins *PDKN1A*, *PDKN1B*, *PDKN2A*, and *TP53*.

The expression of PKCβ and p-AKT, two components of the PIK3/AKT signaling pathway, correlates with adverse outcome.[136,137] Moreover, expression of phosphotyrosine STAT3, enriched in ABC cases, has been associated with inferior outcomes in DLBCL patients. Notably, an 11-gene *STAT3* activation signature has been shown to predict decreased OS, both in the entire DLBCL cohort as well as in the ABC subgroup as described by Huang *et al.*[138] Adverse prognostic significance has also been attributed to the expression of indoleamine 2,3-dioxygenase (IDO)[139] and SKP2.[140,141]

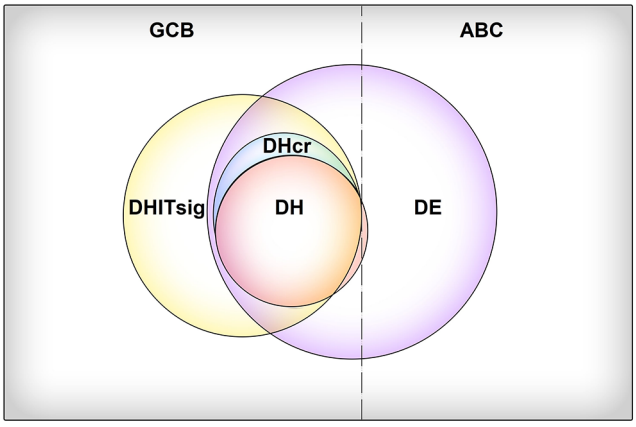

Figure 3. Schematic representation of the distribution of double-hit (DH), double-expressor (DE), and double-hit gene expression signature (DHITsig) diffuse large B-cell lymphoma (DLBCL) cases among the germinal center B-cell (GCB) and activated B cell (ABC) subgroups. DHITsig+ cases, harboring BCL2 and MYC rearrangements cryptic to conventional fluorescence *in situ* hybridization (FISH) are termed cryptic DH (DHcr). The area of each circle corresponds to the relative prevalence of each group among DLBCL cases.

Circulating cell-free DNA

Circulating cell-free DNA (cfDNA) represents DNA fragments released from apoptotic or necrotic cells into the circulation. As DLBCL is characterized by high cell turnover, several studies have evaluated the role of cfDNA in DLBCL prognosis. High levels of cfDNA at diagnosis have been shown to correlate with high tumor burden, advanced stage, high LDH levels, and high IPI score, as well as inferior OS and PFS in DLBCL.[142] In the largest prospective study of 217 DLBCL patients, Kurtz *et al.* demonstrated that cfDNA levels at diagnosis assessed through deep sequencing (CAPP-seq) were predictive of EFS independently of IPI. Applying the same technique, Scherer *et al.*[143] achieved stratification

of DLBCL cases among the COO subgroups, demonstrating high accordance with COO designated by IHC in FFPET. Notably, early decreases in cfDNA 21 days into treatment were highly predictive of the response to R-CHOP and EFS.[144] Global methylation patterns in cfDNA have also been found to predict OS and response to treatment.[145,146] Most importantly, several studies have shown that targeted NGS might be applicable in cfDNA. These studies, apart from validating the prognostic and predictive role of overall cfDNA burden, provide evidence that cfDNA could be used for the genetic characterization of DLBCL cases.[147] Intriguingly, it was recently shown that cfDNA could be used for the stratification of patients in the prognostic genetic subgroups proposed by Wright *et al.*,[45] allowing for an in-depth, minimally-invasive prognostic evaluation of patients.[148]

Therapeutic implications

The addition of rituximab to the standard CHOP regimen has improved survival of DLBCL patients irrespective of COO; however, the ABC subtype still confers adverse prognosis compared with the GCB-subtype, retaining its significant prognostic effect even in the relapsed/refractory (R/R) setting. Therefore, current research focuses on the design of novel therapies that target specific oncogenic pathways which are activated and play a crucial role in the pathogenesis of the disease.

A hallmark of ABC DLBCL is constitutive activation of the *NF-kB* pathway through aberrant BCR signaling and *MYD88* activation.[149] Although thought to represent independent pathways converging to *NF-κB* activation, co-occurrence of *CD79B* and $MYD88^{L265P}$ mutations in a significant subset of ABC DLBCL (namely the MCD subgroup) suggest at a potential interplay between the two pathways. Most recently, the My-T-BCR supercomplex was identified, comprising BCR, MYD88, and TLR9, leading to *NF-κB* and *mTOR* pathway activation.[150]

The significance of the *NF-kB* pathway in the pathogenesis of DLBCL led to the investigation of the proteasome inhibitor bortezomib, which inhibits *NF-κB* by preventing proteasomic degradation of IκBα.[151] Although initial results had been promising, a large randomized phase III trial showed that the addition of bortezomib in the standard R-CHOP did not confer any benefit to the PFS or OS of newly diagnosed DLBCL patients, irrespective of the COO.[152] The disappointing performance of this agent in DLBCL could reflect its unspecific mode of action, highlighting the need for more targeted treatment modalities.

Lenalidomide is an immunomodulatory drug with multiple effects, including inhibition of the *NF-kB* activity through the downregulation of *IRF4* and *SPIB*.[153] Results of the ECOG-ACRIN1412 phase II trial demonstrated that the addition of lenalidomide to R-CHOP could reduce the risk of progression or death by 33%, irrespective of COO. It should be noted that the effect of lenalidomide was more robust within the ABC subgroup.[154] Surprisingly, the ROBUST phase III trial which was based on 570 newly diagnosed ABC DLBCL patients did not show any difference between the lenalidomide-R-CHOP (R^2-CHOP) arm and the arm of standard R-CHOP treatment in terms of PFS.[155] There may be many reasons that explain this difference in the two trials apart from their inherent differences in the study design, such as the higher dosage of lenalidomide in the ACRIN trial or the significantly longer time lag between the diagnosis and initiation of treatment in the ROBUST trial.[156] Nonetheless, lenalidomide may represent a promising agent for tumors within the MCD and BN2 subgroups which consistently overexpress *IRF4*. More studies focusing on these subgroups are needed.

Several components of the BCR pathway have been proposed as potential therapeutic targets in DLBCL. Among them, the inhibition of Bruton's tyrosine kinase (BTK) by ibrutinib is the most well studied. The recently published results of the phase III Phoenix trial, which compares ibrutinib-R-CHOP with R-CHOP for newly diagnosed patients with ABC DLCBL demonstrated that the addition of ibrutinib prolongs PFS and OS in younger (<60 years) patients with ABC DLBCL. The differential effect of ibrutinib by age could be explained by the increased number of serious adverse events in older patients, leading to deviation from treatment schedule or treatment discontinuation.[157] In terms of genetic subgroups, MCD, BN2, and A53 might represent the most BCR-dependent tumors among the ABC subgroup;

therefore, ibrutinib might be particularly beneficial for tumors falling within these subgroups. Notably, co-occurrence of *CD79B* and *MYD88*^{L265P} mutations, a hallmark of the MCD subgroup, predicts high sensitivity to ibrutinib.[158] A recent phase II study of ibrutinib and lenalidomide, in combination with R-CHOP, showed promising results.[159] Other inhibitors of the proximal components of the BCR pathway, such as fostamatinib (syk inhibitor) and enzastaurin (PCKβ inhibitor) have shown limited effect in DLBCL;[160,161] on the other hand, JNJ-67856633, a MALT-1 inhibitor, showed efficacy in preclinical studies and is currently investigated in a phase I trial in DLBCL patients (NCT03900598).[162]

Activation of the *PI3K* pathway represents an important oncogenic event in most DLBCL cases. Within the ABC subgroup, *PI3K* activation occurs mainly as a sequela of BCR activation and leads to *NF-κB* activation; in contrast, in GCB DLBCL it represents the result of *PTEN* inactivating mutations and leads to activation of the *AKT/mTOR* pathway.[163] Idelalisib, a selective PI3Kδ inhibitor, showed disappointing results in DLBCL; however, preclinical data have demonstrated that simultaneous inhibition of PI3Kα and δ is needed to exert cytotoxicity in ABC DLBCL.[164] Consistently, copanlisib, which is a PI3Kα/δ inhibitor, has shown encouraging results as a monotherapy in the R/R setting, particularly for the ABC subgroup.[165] Buparlisib, a pan-PI3K inhibitor, has also been evaluated in a phase II trial; albeit, the effect in DLBCL has been limited.[166] Preclinical data on the synergetic effect of PI3Bα/δ and BTK inhibitors triggered researchers to investigate the efficacy of the combination of PI3K inhibitors and ibrutinib.[167] For MK-2206, an AKT inhibitor which had shown promising results in preclinical models, the results in the clinical setting have been rather disappointing.[168] Regarding mTOR inhibitors, everolimus and temsirolimus have demonstrated activity in the R/R setting; however, in the frontline setting, adjuvant therapy with everolimus after R-CHOP did not improve the disease-free survival (DFS) of high-risk patients.[169] Recently, a phase I trial evaluated the safety of everolimus in combination with R-CHOP for newly diagnosed DLBCL; although the combination has been deemed safe, its superiority to standard R-CHOP treatment has not yet been evaluated.[170] In conclusion, more studies are needed to evaluate the effect of PI3K/mTOR inhibitors in DLBCL. It should be noted that, based on GEP studies, the MCD, BN2, ST2 and EZB subgroups might benefit more from this therapeutic approach.[45]

BCL2 plays an essential role in DLBCL pathogenesis, particularly within the MCD, BN2, and EZB genetic subgroups. In this context, venetoclax, a selective BCL2 inhibitor, has been evaluated in DLBCL. A recent phase II study of 208 newly diagnosed patients demonstrated that the addition of venetoclax to R-CHOP provided improved OS and PFS compared with standard treatment. Notably, venetoclax was effective even in cases not expressing BCL2, although the effect was more robust in BCL2+ patients.[171] Based on this finding, venetoclax might be beneficial in the treatment of DH/TH lymphomas, although this should be confirmed by randomized trials.

The *JAK/STAT* pathway is also implicated in the pathogenesis of a subset of DLBCL, corresponding to the MCD and ST2 genetic subgroups. JAK inhibition might represent a promising treatment approach in this subset.[45] A preliminary phase I trial has shown modest efficacy of pacritinib, a JAK1/2 inhibitor, in R/R DLBCL patients.[172]

The finding that *EZH2* is mutated in up to 22% of GCB DLBCLs, comprising the EZB subgroup, has drawn attention to the role of hypomethylating agents in DLBCL treatment.[173] An EZH2 inhibitor called tazemetostat has shown promising results. Interim results of a phase II trial in R/R DLBCL showed an ORR of 40% in patients with DLBCL harboring EZH2 mutations, compared with 18% in patients with wild-type *EZH2*.[174] A phase I trial has also shown the feasibility and safety of tazemetostat in combination with R-CHOP in the frontline setting.[175]

In contrast to Hodgkin lymphoma and solid tumors, checkpoint inhibitors have yielded disappointing results in NHL, potentially because of the low prevalence of PD-L1 overexpression in DLBCL.[176] However, checkpoint inhibitors combined with other agents might be effective in a subset of DLBCL patients with high PD-L1 expression. Durvalumab has recently been evaluated in combination with R-CHOP or R^2-CHOP for the frontline treatment of high-risk patients, including a considerable number of DH/TH. The combination demonstrated its efficacy and safety,

but randomized phase III trials are needed to establish its efficacy.[177] A potential evidence-based approach for treatment selection, which takes into account the molecular subgroups of DLBCL, is presented in Table 3.

Therapeutic approach for DH/TH lymphomas

Based on their aggressive nature, DH/TH lymphomas require a more intensified therapeutic approach. A meta-analysis of retrospective studies compared with OS and PFS of DH lymphoma patients treated with the standard R-CHOP on the one hand, to more intensified treatment protocols such as dose-adjusted R-EPOCH (rituximab, etoposide, doxorubicin, cyclophosphamide, vincristine, prednisone), Hyper-CVAD, and R-CODOX-M/IVAC on the other. Treatment with dose-adjusted R-EPOCH yielded a median PFS of 22.2 months, compared with 12.1 months with R-CHOP, as well as a 34% reduction in progression-risk; however, no effect on OS was noted.[178] Most recently, the phase III ALLIANCE trial did not demonstrate a survival benefit for patients treated with dose-adjusted R-EPOCH compared with treatment with R-CHOP; however, *MYC*-rearranged cases, and DH/TH cases were significantly underrepresented within the study population. Therefore, extrapolation of the results in this subgroup would not be advised.[179] Nonetheless, the results of a phase II trial on MYC-rearranged DLBCL cases showed promising results, with 2-year PFS and OS of 71% and 76.7% respectively.[180] Given the lack of randomized trials focusing on DH/TH HGBL, dose-adjusted R-EPOCH represents an encouraging frontline treatment approach for these patients.

Other agents have been tried in order to mitigate the inferior prognosis of DH/TH. Venetoclax, in combination with dose-adjusted R-EPOCH, has been evaluated in a phase I trial which demonstrated acceptable safety and efficacy, leading to its current evaluation in a phase II/III trial.[181] Tazemetostat and other epigenetic modulators might also prove effective in the treatment of DH/TH. Other novel agents, such as bromodomain, and external domain (BET) inhibitors and Aurora kinase inhibitors might also be effective, as they work by disrupting downstream *MYC* signaling. These agents are still in preclinical or early clinical trials.[182,183] The therapeutic agents that might be effective in the subset of DH/TH are summarized in Table 4.

Conclusions

Several lines of evidence have been published with regards to the prognostic biomarkers of DLBCL in the rituximab era. Past established prognostic factors have been disputed in the rituximab era, whilst there are still conflicting data on the prognostic value of innovative biomarkers. The retrospective nature of most studies, the lack of validation within large prospective trials, the lack of reproducible techniques, and the use of different cut-offs (especially regarding certain IHC markers) are some of the reasons that studies have failed to reflect the underlying complexity of the disease pathophysiology. Moreover, significant inter-correlation of individual biomarkers as well as correlation between biomarkers and IPI categories confound the results of the studies. In the effort to evaluate these prognostic biomarkers, a great variety of methods, including IHC, GEP, NGS, and genomic hybridization have been trailed, but very few are applicable in clinical practice due to cost-related factors and lack of reproducibility.

Among the evaluated prognostic biomarkers, COO, concurrent rearrangements of *MYC*/*BCL2*/*BCL6*, the characterization of DH/TH HGBL, and the overexpression of *MYC*/*BCL2*, characterizing DE lymphomas, remain the more robust tools to identify high-risk patients that might need treatment intensification and incorporation of novel target treatment modalities. However, it should be acknowledged that most studies have failed to demonstrate a survival benefit by differentiating the therapeutic approach in these patients. The wide genetic heterogeneity of tumors, even within the same COO subgroup, might explain why individualized treatment simply based on COO classification has providing disappointing results. Most recently, GEP studies have managed to partially elucidate the complex genetic and transcriptomic landscape of DLBCL identifying gene-expression signatures, allowing for the classification of DLBCL cases in prognostically relevant genetic subgroups. The same method has been employed for disentangling the complex composition of the TME and elucidating its prognostic significance. Efforts to translate the results of these studies into techniques applicable

Table 3. Summary of major prognostic biomarkers in DLBCL.

Prognostic factor	Effect on prognosis	Comments
Cell of origin (COO)		
ABC by GEP, Lymph2Cx[7–19]	UF	
Non-GCB by IHC[20–31]	UF#	Inferior to GEP in prognostication
LMO2[36–40]	F	
Molecular subgroups[45]		
MCD, N1, A53	UF	
BN2, ST2	F	
EZB	F, if DHITsig-negative	
	UF, if DHITsig-positive	
Somatic hypermutation subgroups[46]		
SHM1, SHM2	UF	
SHM3, SHM4	F	
BCL2		
Overexpression[76,77]	UF#	In absence of *MYC* overexpression: no effect on OS
Rearrangement[74,75]	UF#	In absence of *MYC* rearrangement: no effect on OS
MYC		
Overexpression[85]	UF#	In absence of *BCL2* overexpression: no effect on OS
Rearrangement[80–85]	UF#	In absence of *BCL2* rearrangement: no effect on OS
BCL6		
Overexpression[89,90]	F#	Strong correlation with ABC subgroup: potential confounder
Rearrangement[88]	UF#	Strong correlation with GCB subgroup: potential confounder
DH/TH[82,85,93,94]	UF	The role of DH-BCL6 is equivocal.[97–110]
		Non-IG partner gene in MYC rearrangement: no effect in OS[101]
Double-expressor[85,86,107]	UF	
DHITsig/MHG[103–105]	UF	
TP53		
Mutations[109–111]	UF	
Overexpression[109,113]	UF#	Overexpression in the absence of TP53 mutation: No association with OS
CD5[120–122]	UF	
Low CD20[123–125]	UF	

(continued)

Table 3. (Continued)

Prognostic factor	Effect on prognosis	Comments
CD30[126]	F#	Potential role of brentuximab vedotin, UF in EBER-positive cases
Ki-67[114]	UF	
TME composition		
GB-like, MS subgroups[70]	F	
IN, DP subroups[70]	UF	
Stromal-2 expression[9]	UF	
Stromal-1 expression[9]	F	
High CD4*CD8:M2*PD-L1 ratio[69]	F	
High LAMIS expression[56]	UF	
VEGFR2/VEGFR1[47,48]	UF	
HIF-1a[49]	F	
SPARC[50,51]	F	
MHC-II loss[65–68]	UF	
PD-L1 (expressed by tumor cells)[59–61]	UF	Potential role of immune checkpoint inhibitors
PD-1 (expressed in TME)[62,63]	F	
FOXP3[53,54]	UF#	
Cell-cycle regulation and apoptosis		
BCL2L12[127]	UF	
BIRC5[129]	UF	
XIAP[128]	UF	
Other		
*PKC*β[137]	UF	
p-AKT[136]	UF	
STAT3[138]	UF	
Circulating cell-free DNA[142–148]	UF	

#Studies show conflicting results regarding the prognostic effect of this biomarker.
ABC, activated B-cell; COO, cell of origin; DH/TH, double/triple-hit lymphomas; DHITsig, double-hit signature; DLBCL, diffuse large B-cell lymphoma; DP, depleted; F, favorable; GB-like, germinal center-like; GCB, germinal center B-cell; GEP, gene-expression profiling; IG, immunoglobulin; IHC, immunohistochemistry; IN, inflammatory; LAMIS, lymphoma-associated macrophage interaction signature; MHG, molecular high-grade; MS, mesenchymal; OS, overall survival; TME, tumor microenvironment; UF, unfavorable.

Table 4. Potential therapeutic agents by molecular subgroup of DLBCL[45]. Potential therapeutic approaches for double/triple-hit lymphomas (DH/TH) are also noted.

Subgroup	Potential therapeutic agents
BN2	BTK inhibitors (ibrutinib, acalabrutinib, zanibrutinib)
	Lenalidomide
	PI3K/mTOR inhibitors (copanlisib, buparlisib, everolimus)
A53	BTK inhibitors
ST2	JAK/STAT inhibitors (ruxolitinib, pacritinib)
	PI3K inhibitors
MCD	BTK inhibitors
	Lenalidomide,
	JAK/STAT inhibitors
N1	Immune checkpoint inhibitors (nivolumab, pembrolizumab, durvalumab)
EZB	EZH2 inhibitors (tazemetostat)
	PI3K inhibitors
	BCL2 inhibitors (venetoclax)
DH/TH	R-da-EPOCH (rituximab, dose-adjusted etoposide, vincristine, cyclophosphamide, doxorubicin, prednisone)
	BCL2 inhibitors
	EZH2 inhibitors
	PI3K inhibitors

BCL2, B-cell lymphoma 2; BTK, Bruton's tyrosine kinase; DH/TH, double/triple-hit; EZH2, enhancer of zest homolog 2; JAK, Janus kinase; mTOR, mechanistic target of rapamycin; PI3K, Phosphoinositide-3 kinase; STAT, signal transducer and activator of transcription.

in clinical practice are being made. In this context, Lymph2Cx, the gold standard for COO determination, can be expanded to allow for identification of DHITsig+ cases in true need of a more intensified treatment approach. Similarly, targeted NGS can be used to stratify patients among the novel genetic subgroups that might benefit from specific novel agents. Notably, these techniques have been validated for application in FFPET; therefore, their use can be expanded in clinical practice. More intriguingly, liquid biopsies and targeted NGS in cfDNA might revolutionize prognostication in DLBCL. Genetic characterization of cases, and classification in COO and genetic subgroups through studies in cfDNA might overcome limitations pertaining to the quantity and quality of biotic samples and allow for the evaluation of dynamic changes in the genetic landscape of DLBCL during treatment and follow-up. Considering the TME, the translation of the recent findings into clinically applicable methods for stratifying patients in prognostic subgroups is eagerly anticipated.

As knowledge regarding the complex genetic landscape of DLBCL accumulates, the ultimate goal is a comprehensive evaluation of the gene-expression and mutational profile, both in the tumor cells and TME of each DLBCL case, which might allow for more precise prognostication and provide the basis for individually-tailored treatment of DLBCL patients.

Conflict of interest statement

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