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PERSPECTIVE

Is now the time for molecular driven therapy for diffuse large B-cell lymphoma?

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ABSTRACT

Introduction: Recent genetic and molecular discoveries regarding alterations in diffuse large B-cell lymphoma (DLBCL) deeply changed the approach to this lymphoproliferative disorder. Novel additional predictors of outcomes and new therapeutic strategies are being introduced to improve outcomes.

Areas covered: This review aims to analyse the recent molecular discoveries in DLBCL, the rationale of novel molecular driven treatments and their impact on DLBCL prognosis, especially in ABC-DLBCL and High Grade B Cell Lymphoma. Pre-clinical and clinical evidences are reviewed to critically evaluate the novel DLBCL management strategies.

Expert commentary: New insights in DLBCL molecular characteristics should guide the therapeutic approach; the results of the current studies which are investigating safety and efficacy of novel 'X-RCHOP' will probably lead, in future, to a cell of origin (COO) based upfront therapy. Moreover, it is necessary to identify early patients with DLBCL who carried MYC, BCL2 and/or BCL6 rearrangements double hit lymphomas (DHL) because they should not receive standard R-CHOP but high intensity treatment as reported in many retrospective studies. New prospective trials are needed to investigate the more appropriate treatment of DHL.

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1. Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most frequent type of adult non-Hodgkin lymphoma (NHL) in Western countries, comprising 25–30% of all cases of NHL [1]. In the United States, about 24,000 new cases/years are estimated to have been diagnosed in 2015 [2].

Benefits in the outcome of patients with DLBCL have been achieved following the introduction of the anti-CD20 monoclonal antibody rituximab in the therapeutic algorithms. The first positive experiences with rituximab in association with the standard CHOP regimen (adriamycin, cyclophosphamide, vincristine, prednisone) were observed in elderly patients (60–80 years) and then further confirmed in young patients (<60 years) by the MInT trial that led FDA to approve the use of rituximab as first-line treatment for DLBCL in 2006 [3,4].

Despite these improvements, approximately 40% of DLBCL patients will relapse or have a refractory disease. The use of potentially more intensive chemotherapies has failed to demonstrate a higher efficacy compared to R-CHOP. Several randomized studies using autologous stem cell transplantation (ASCT) in first remission or dose-dense strategies have failed to document a clear advantage over R-CHOP [5,6].

For these reasons, clinical and translational researchers have tried to identify clinical and/or biologic hallmarks with the aim of better stratifying patients at high risk of relapse/refractoriness with standard therapy. The International Prognostic Index (IPI) score currently in use is easy to perform but does not take into consideration the novel biologic acquisitions generated in the last years [7].

The common feeling that DLBCL is a complex neoplastic disease is clearly underlined by the differences in clinical presentation, morphology, genetic, and molecular profiles. The only recognition of morphologic variants (e.g. immunoblastic DLBCL) has failed to define a valid correlation with clinical outcome, probably because influenced by the observers' experience and interpretation. However, among the RICOVER-60 trial, the German High-Grade Lymphoma Study Group (DSHNHL) tested the prognostic impact of immunoblastic morphology in 949 patients. In this analysis, the authors showed that the immunoblastic morphology was an adverse prognostic factor in multivariate analysis [8], furthermore, another study revealed that the immunoblastic variant of DLBCL has a high frequency of underlying *MYC* translocation [9].

The translational studies of DLBCL have allowed to identify different patterns of gene expression profile (GEP) which correlate with clinical outcome. In 2000, Alizadeth et al. [10], using DNA microarrays, showed that there are different GEPs among the tumors of DLBCL patients. They identified two molecularly distinct subtypes of DLBCL based on the cell of origin (COO). One subtype expressed genes characteristic of germinal center B-cells (germinal center B-cell like DLBCL or GCB-DLBCL); the second subtype expressed genes normally induced during *in vitro* activation of peripheral blood B cells (activated B-cell like DLBCL or ABC-DLBCL). This means that DLBCL can arise from different cell types in the microenvironment of the lymph node. Approximately 15–20% do not fit into these two categories and are molecularly unclassifiable

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[11]. Different immunohistochemistry (IHC) methods are also used to classify DLBCL according to COO, using these algorithms, DLBCL is classified as GCB or non-GCB [12–14].

The GCB-DLBCL is associated with a favorable outcome when treated with standard RCHOP therapy; however, the better understanding of the molecular pathogenesis of the two subgroups has led to investigation of new targeted therapeutic strategies to improve the worse outcome of the patients with ABC/non-GCB DLBCL.

The importance of recognizing the two biologic categories has been highlighted in the most recent World Health Organization (WHO) revision of lymphoma classification [15], which considers acceptable to use the IHC algorithm since GEP is not routinely utilized. It must, however, be underlined that IHC is not able to classify the 15% of patients identified by GEP as unclassifiable.

Newer methods based on quantification of RNA transcripts extracted from formalin-fixed paraffin-embedded tissues have concordant results with conventional microarray GEP and may overcome this difficulty in COO classification [16].

In addition to the COO, recent genetic and proteomics studies identified a prognostic role for *MYC* and *BCL2* genetic translocations and/or protein co-expression. Earlier studies reported that 5–10% of DLBCL harbored *MYC, BCL2*, and/or *BCL6* translocations, and were named as 'double hit' lymphoma (DHL), or triple hit lymphoma (THL). The new revised WHO lymphoma classification recognized this category as the new entity "high-grade B-cell lymphoma (HGBL) with rearrangements of *MYC, BCL2* and/or *BCL6''[15]*.

A comprehensive understanding of the mutations which drive DLBCL pathogenesis is not still clear; whole-exome sequencing could have a role in the identification of significant gene mutations. Lohr et al. demonstrated in a small series of patients (DLBCL) that the mutations of three genes – KRAS, BRAF, and NOTCH1 – have a role in the oncogenesis of DLBCL. If those data will be confirmed in a larger number of patients, it could be possible to expand our knowledge regarding DLBCL over the simple COO [17].

The aim of this review is to analyze the recent molecular discoveries in DLBCL biology and the subsequent potential new treatment options.

1.1. Beyond IPI: new prognostic factors in DLBCL

The prognostic factors in DLBCL can be divided into those related to the patient (e.g. age and performance status), those related to the tumor (e.g. stage, tumor burden, proliferating fraction, extranodal involvement), those related to aggressiveness indicators (e.g. serum lactate dehydrogenase (LDH) and beta-2-microglobulin levels). However, considering the advanced knowledge on the molecular bases of deregulated signaling in DLBCL, new factors related to the complex biology of the disease need to be added (Figure 1).

IPI is routinely used to predict the outcome of patients with DLBCL and remains a robust prognostic tool [7]. IPI was originally created in the pre-rituximab era and it was based on a combined score of five clinical parameters: age > 60, elevated serum LDH, ECOG performance status ≥ 2, Ann Arbor stage III or IV, number of involved extranodal sites ≥ 2, which reflect the extent of the tumor involvement and the host status. Based on this score, four risk categories (low, low-intermediate, high-intermediate, and high) have been identified with 5-year overall survival (OS) of 73%, 51%, 43%, and 26%, respectively. To stratify patients 60 years or younger, the age adjusted IPI, which includes stage, LDH, and performance status, is more commonly used in the clinical practice than IPI [18]. Sehn et al. [19] confirmed the prognostic value of the

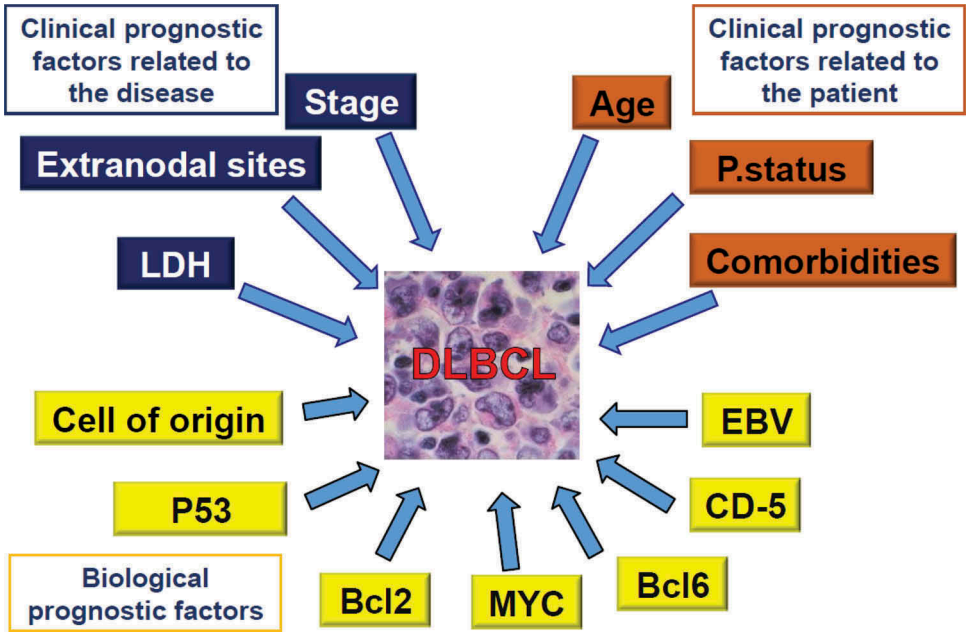

Figure 1. Clinical and biological factors affecting response in DLBCL.
LDH - lactate dehydrogenase.

IPI score in a cohort of R-CHOP-treated patients, but they were able to distinguish only two risk group instead of the four ones of the original IPI. They subsequently proposed a revised version (R-IPI) that considered three risk groups with 4-year OS rates of 94%, 79%, and 55%, respectively. The German group analyzing the data from three prospective phase II/III trials demonstrated that the conventional IPI has still a prognostic relevance in the R-CHOP era [20].

With the aim to improve the risk stratification by IPI in Rituximab era, a new IPI was built using raw clinical data from the National Comprehensive Cancer Network (NCCN) database: the NCCN-IPI. This new score system better discriminated low- and high-risk subgroups (5-year OS: 96% vs. 33%) than the IPI (5-year OS: 90% vs. 54%), respectively [21].

To date, we are in the molecular era of disease definition and even though the IPI score remains a robust prognostic index, it does not address the underlying biologic heterogeneity of DLBCL. Additional predictors are necessary to refine the prognostic stratification and identify more precisely high-risk patients.

DLBCL is characterized by highly heterogeneous genetic and cytogenetic aberrations. Some alterations are frequently found in DLBCL, probably playing an important role in lymphomagenesis or progression of the disease. These include rearrangements and mutation involving *BCL2, c-MYC, BCL6,* and *TP53*, and many of these biological parameters have been identified as prognostic factors in DLBCL [22] (Table 1).

1.1.1. Cell of origin

As described above, the molecular evaluation of the COO has identified two major subtypes with a different outcome: the GCB-DLBCL and the ABC-DLBCL. Notably, this remarkable biologic difference was also associated with a different prognostic likelihood: GCB-DLBCL presented a significantly better outcome when treated with R-CHOP, with a 5-year OS of 76% compared to 34% in ABC-DLBCL ($p < 0.01$) as reported in the Hans et al. study. In the same study, in multivariate analysis, a high IPI score (3–5) and the non-GCB phenotype were independent adverse predictors ($p < .0001$) [12]. Hans et al. suggested an IHC algorithm which evaluates the expression of three cellular proteins: CD10, BCL6, and MUM1/IRF4 in identifying GCB or non-GCB type; however, this algorithm shows only a 79% of concordance with GEP [12]. The introduction of other IHC markers like GCET1 and FOXP1 enhanced the sensitivity of IHC in the definition of the COO; a higher grade of concordance between IHC and GEP (93%) was found by Choi et al., which considered GCET1, CD10, BCL6, MUM1, and FOXP1 markers [13]; a similar grade of concordance (92.6%) between GEP and IHC was also demonstrated by Visco et al. by using CD10, GCET1, FOXP1, BCL6, and MUM1 evaluation algorithm [14]. However, there were discrepancies in the clinical outcomes predicted by these different algorithms and, to date, none of them was robust to identify the DLBCL COO compared to the gold standard GEP classifier [23–25]. Lenz et al. profiled gene expression signatures in pretreatment biopsy of 181 patients with DLBCL treated with CHOP and 233 patients treated with R-CHOP. They demonstrated that even in the RCHOP cohort, the patients with GC-DLBCL had higher OS rates than those with ABC-DLBCL, 74% vs. 40% [26]. These data suggest that the identification of the molecular profile of DLBCL can further refine the traditional prognostic score derived from the clinical assessment of patients; however, the difficulties of using GEP analysis has limited the use of this method in the daily clinical practice.

Recently, RNA-based new technologies have enabled to quantify the level of expression of the most significant genes of the GCB/ABC signature, in formalin-fixed paraffin-embedded tissues (FFPET). One of the two major platforms developed, named NanoString (Lymph2Cx), has a rapid turn-around time of 36 h and recent reports indicate that this assay may allow to identify more robustly the COO than the widely used algorithm of Hans et al [16]. When applied to a large cohort of DLBCL treated with RCHOP, the Lymph2Cx signature was able to classify GCB versus ABC DLBCL, and the prognosis of ABC type was poorer as expected [27]. No study has formally compared different gene set Nanostring signatures to date. The feasibility and accuracy of the Nanostring technology is being assessed among a prospective trial (ROBUST trial). This technology seems the most advanced to date in terms of validation against GEP gold standard and reproducibility between laboratories.

In addition, in many medical centers high-throughput sequencing technologies (next-generation sequencing [NGS]) are becoming currently available and are be able either to identify specific target mutations either to assess mRNA expression levels from FFPE samples at limited costs. In the near future, we could have the combination of gene expression level and targeted sequencing of mutations (such as *MYD88, GNA13*) as benchmark.

Beyond GEPs, genomic gains and losses of specific oncogenes or tumor suppressors, as *MYC* and *CDKN1B* gains or *TP53* and *CDKN2A* losses, were significantly associated with outcome independently of the ABC and GCB subtypes [28].

1.1.2. CD5

CD5 is an antigen typically expressed on the surface of normal and neoplastic T cells, as well as on a subset of normal IgM-secreting B cells (B1 repertoire) and of neoplastic B cells [29].

De novo CD5+ DLBCLs represent a distinct subgroup of DLBCL first described in 1995, that occurs in approximately 5–10% of newly diagnosed cases and that shows an aggressive clinical course and poor prognosis [30,31]. Common characteristics of patients with CD5+ DLBCL are older age, a high/high-intermediate IPI at diagnosis, frequent extra-nodal involvement, and an ABC phenotype. Most patients show a primary refractory disease or relapses after front-line chemotherapy. So far, there is no consensus on the optimal first-line treatment for CD5+ DLBCL [32]. In one of the largest series of *de novo* CD5+ DLBCLs diagnosed and treated in Western countries, Alinari et al. [33] retrospectively reviewed the clinical features and outcome of 102 patients with *de novo* CD5+ DLBCL treated with rituximab plus chemotherapy. The 3-year progression-free survival (PFS) for the ABC and GCB subtypes was 34% and 45%, respectively, while the 3-year OS for ABC and GCB-subtypes was 62% and 67%, respectively. These data suggest that CD5+ DLBCL patients have a poor outcome despite the COO.

1.1.3. Epstein–Barr virus (EBV)

Epstein–Barr virus (EBV)-positive lymphoproliferative disorders are more common in patients with congenital or acquired immunodeficiency disorders. Oyama et al. [34] were the first to describe EBV-positive DLBCL (E-DLBCL) in immunocompetent patients. These types of lymphomas are frequently diagnosed in elderly patients and are characterized by an aggressive clinical course, poor response to chemotherapy, high rate of relapse after first-line treatment, and decreased OS, even in the rituximab era [35,36]. Age-related senescence of the immune system seems to be one of the most important factors in the pathogenesis of E-DLBCL causing an impaired cytotoxic T-cell immune surveillance toward EBV [37]. The 2008 WHO classification of lymphoid neoplasm classified this disease in a provisional entity as EBV-positive DLBCL of the elderly, defined as a clonal EBV+ B-cell proliferation occurring in immunocompetent patients with more than 50 years.

Despite the name, recent reports have shown that this entity can also affect younger patient;, however, in the Hong et al. study, the EBV positivity did not affect the OS in the young patients (<50 years) but remained a predictor of inferior survival in the patients with more than 50 years [36]. Recently, in another study, Lu et al. investigated the outcome of EBV-positive patients in different age cohorts using different EBER cut-offs. Using both the 20% and 50% EBER cut-off values, they found that EBV-positive patients showed a significantly worse OS and PFS than the negative cases, irrespective of age [38].

1.1.4. TP53

TP53 is a cellular protein encoded by homologous genes localized in the short arm of chromosome 17. It has a crucial role in the regulation of cell cycle, DNA repair, apoptosis, and senescence with an important tumor suppression activity [39]. Several studies have shown that different types of *TP53* mutations are an independent prognostic factor of poor outcome in newly diagnosed DLBCL treated with CHOP chemotherapy with or without rituximab [40–42].

Young et al [43] in 2008 found a strong association between *TP53* mutations (*MUT-TP53*) and clinical features such as younger age at diagnosis, high serum LDH levels, bulky mass, and a high IPI risk group. They also found that *TP53* mutations prognostically stratify only the GCB-DLBCL group into distinct risk subsets. In the series by Xu-Monette et al. [42], a large cohort of newly diagnosed DLBCL (506 patients) treated with R-CHOP was evaluated for *TP53* gene mutations by exon sequencing. The 5-year OS was 65.9% for patients with wild type *TP53* (*WT-TP53*) compared to 47.8% for those with *MUT-TP53* DLBCL. The 5 year PFS was 63.5% for *WT-TP53* patients versus 46.3% for *MUT-TP53* cases. In contrast with previous studies, in this series *TP53* mutations seem to impact on survival in both GCB and ABC subtypes of DLBCL, with a major impact on GCB DLBCL. They also evaluated TP53 overexpression by IHC as a surrogate of *TP53* mutation and it proved to be a good predictor of mutation when a cut-off greater than 50% is used.

Interestingly, Gebauer et al. [44] in a recent retrospective study found an elevated frequency of *TP53* mutations in newly diagnosed DHL with *MYC* and *BCL2* aberrations, but not in *MYC-BCL6* DHL. The prognostic effect of this association was not evaluated.

1.1.5. BCL6

BCL6 is a zinc finger transcriptional factor encoded by a proto-oncogene localized on chromosome 3. This protein acts as a transcription repressor of many genes with a major role in GC formation and in the regulation of lymphocyte differentiation and survival [45]. A deregulated expression, due to translocations or mutations, has been implicated in the lymphomagenesis process [46].

It has been demonstrated that the *BCL6* gene is one of the most frequently translocated or mutated genes in DLBCL. In fact, its rearrangement occurs up to 30–35% of newly diagnosed cases leading to a deregulation of the expression and function of the BCL6 protein [47,48]. The effect of *BCL6* translocation or hypermutation on prognosis remains uncertain; different results have been reported, some associated with a favorable prognosis [49,50], others with an unfavorable outcome [47,51] and others with no impact at all [52].

In the pre-rituximab era, BCL6 protein expression had been identified as one of the strongest predictors of outcome in DLBCL [53]. In fact, patients with a high expression of BCL6 were the ones showing the longer OS, but this favorable prognostic role of BCL6 expression was not confirmed when rituximab was added to CHOP [51].

GCB-DLBCL shows in general a high expression of BCL6 by IHC and is associated with a better OS than the ABC subgroup which does not show a BCL6 overexpression.

In the series by Iqbal et al. [50], the authors demonstrated that the translocation occurs mostly in the ABC subtype, while gene mutations occur mostly in the GCB subtypes. In their cohort of patients, BCL6 overexpression, evaluated by IHC at a 30% cut-off, was significantly associated with a favorable OS despite the COO. Also, higher levels of *BCL6* mRNA were associated with a better outcome in the whole cohort.

In contrast, Akyurek et al. [49] reported on 239 patients with DLBCL evaluated for *BCL6, BCL2,* and *MYC* rearrangements. They found that a *BCL6* rearrangement had a significantly negative impact on survival in patients with the non-GCB phenotype, while there was no impact in patients with GCB-DLBCL. Also, BCL6 did not affect PFS. In contrast with previous studies, they did not find a correlation between *BCL6* rearrangement and BCL6 expression in IHC [46,52].

The extreme heterogeneity of these results could be explained by the absence in most studies of simultaneous *MYC* rearrangements assessment. More data on *BCL6* rearrangements, as single genetic alteration, are needed to conclusively define the impact on OS and PFS for a better risk stratification of patients at diagnosis.

1.1.6. BCL2

The BCL2 protein is a mitochondrial outer-membrane protein encoded by the *BCL2* proto-oncogene localized on chromosome 18. It plays a critical role in promoting cell survival and inhibition of proapoptotic proteins such as BAX and BAK [53].

Its expression is frequently found abnormal in human cancers such as lymphomas and leukemia. High expression levels of BCL2 in neoplastic cells have been associated with resistance to chemotherapy agents [54].

BCL2 is frequently found overexpressed in both the ABC and GCB DLBCL subtypes. Up to 35% of GCB DLBCL shows a *BCL2* rearrangement with a t(14;18) (q32;q21), which is associated with high *BCL2* mRNA levels and high expression of the BCL2 protein [54,55]. In a few cases, *BCL2* is translocated to an Ig light chain (IGκ, IGλ) locus, as in t(2;18)(p11;q21.3) or t(18;22)(q21.3;q11). *BCL2* translocation is rarely found in DLBCL with an activated phenotype, suggesting that the overexpression of BCL2 found in this subset has an alternative pathogenesis [56]. The constitutive activation of the NF-kB pathways [57] is the pathogenetic hallmark of ABC DLBCL which leads to an increased transcription of the *BCL2* gene; the other mechanism that may explain the overexpression is the amplification of the locus 18q21 [58] on which *BCL2* resides and that is found in particular in ABC DLBCL [59].

Recent studies conducted in cohorts of patients treated with R-CHOP have evaluated the prognostic significance of BCL2 expression. The results still remain conflicting, possibly due to the different cut-off levels used for the IHC analysis and due to the heterogeneity of the DLBCL cohorts with variable proportions of GCB and ABC DLBCL. In some series, BCL2 expression did not predict for either PFS or OS since the addition of rituximab to CHOP chemotherapy seemed to eliminate the gap between BCL2-positive and BCL2-negative patients [60,61] reported in the pre-rituximab era [62,63]. Iqbal et al. [64] evaluated BCL2 expression (with a 50% cut-off for IHC analyses) in 221 patients treated with R-CHOP demonstrating that BCL2 protein expression was significantly associated with a poorer OS ($p = 0.009$) and event-free survival (EFS; $p = 0.001$). When patients were divided according to COO by GEP analysis, BCL2 expression had an impact on outcome only in GCB patients. In fact, patients with a GCB phenotype and BCL2 expression had a significantly lower OS ($p = 0.03$) and EFS ($p = 0.002$) than those with BCL2-negative GCB DLBCL.

Visco et al. [65] in 2013 found that, as expected, amplification of *BCL2* was more frequently associated to an ABC phenotype (70%, $p < 0.0001$), while t(14;18) was more associated to a GCB phenotype (84%, $p < 0.0001$). They found no impact on survival of *BCL2* rearrangements on the whole DLBCL cohort, but when patients were stratified according to the COO (evaluated by GEP), patients with GCB-DLBCL and isolated *BCL2* translocations had a significantly worse outcome than patients without *BCL2* rearrangements ($p = 0.0002$) irrespective of any *MYC* rearrangement. Their outcome was similar to that of patients with ABC-DLBCL ($p = 0.30$). They also evaluated the prognostic significance of BCL2 overexpression and confirmed the association with an inferior outcome in GCB patients.

1.1.7. MYC

MYC (c-MYC) is a proto-oncogene sited in the long-arm of chromosome 8 that encodes for a multifunctional transcription factor that plays a critical role in cell cycle progression, differentiation, metabolism immune response, and apoptosis [66]. Mutations, rearrangements, and translocations of this gene have been associated with a variety of hematopoietic tumors such as Burkitt lymphoma, DLBCL, and acute lymphoblastic leukemia. Overexpression of the MYC protein can be detected in a large proportion (about 30–50%) of DLBCL and it is highly expressed (>70% of cells) in the nuclei of DLBCL with *MYC* rearrangements or amplification [67]; not all *MYC* translocations result in MYC protein expression [68]. Only one-third of DLBCL cases with substantial (>30–40% cells) MYC protein expression carry *MYC* gene alterations [67,69,70].

MYC deregulated expression in lymphoma most commonly results from a chromosomal translocation. However, other mechanisms such as amplification, mutation, or microRNA-dependent mechanisms can lead to increased MYC protein expression [71]. The t(8;14)(q24;q23) translocation or, more rarely, t(2;8)(p12;q24) and t(8;22)(q24;q11) that juxtapose the *c-MYC* gene to the immunoglobulin heavy chain (IgH)/light chains (Igκ/λ) gene promoter, cause a high level of transcription of the *MYC* gene. It can be detected in almost 5–10% of DLBCL [69,72]. In 20–30% of *MYC*-translocated DLBCL, it is also possible to find the coexisting *BCL2* and/or *BCL6* rearrangements determining the DHL or THL [22].

The prognostic significance of MYC overexpression in newly diagnosed DLBCL has been evaluated by IHC analysis only in a few studies. The optimal cut-off point for MYC expression is controversial, ranging from 40% to 70% in most studies. Valera et al. [70] in their cohort of 196 patients treated mostly with R-CHOP found that MYC overexpression frequently correlated with older age, advanced stage disease, high serum LDH concentration, and high-risk IPI score, and that it was associated with an unfavorable impact on both PFS and OS ($p = 0.007$) even with a cut-off threshold of 10%.

In a recent paper, Xu-Monette et al. [73] determined the presence of MYC expression by IHC ($n = 535$) and *MYC* rearrangement status by FISH analysis ($n = 344$) in a well-characterized DLBCL cohort. They found that the MYC expression level was significantly higher in ABC-DLBCL than in GCB-DLBCL. In particular, using a 70% threshold for positivity, 37.8% of ABC and 27.9% of GCB-DLBCL showed a high expression of MYC, while a translocation/rearrangement occurred more frequently in GCB-DLBCL (16.3% vs. 7.3%, respectively). GCB-DLBCL patients with a concomitant MYC overexpression and translocation had a significantly worse OS compared to GCB-DLBCL patients with low MYC expression. In ABC-DLBCL, only patients with overexpression but without gene rearrangements had a significantly worse OS, especially if associated with a BCL2 overexpression. Interestingly, *MYC* rearranged DLBCL without MYC protein overexpression did not show a significantly worse survival.

Tzankov et al. [74] evaluated *MYC* rearrangements by FISH in a cohort of patients treated with R-CHOP and confirmed that within the *MYC* rearranged cases (39/376) a higher frequency was found in GCB-DLBCL (27/39; 69%). All types of *MYC* rearrangements (except for amplification which had no impact) were associated with a poor disease-specific survival. Regarding the prognostic role of the MYC protein expression, the expression of the MYC protein in up to 75% or in up to 95% (the most relevant predictive score of *MYC* rearrangements) of tumor cells was associated with an adverse OS; no significant survival difference

has been observed when other cut-off scores have been used (50% or 40%) between MYC protein positive and negative cases.

It is clear that the evaluation of MYC expression and rearrangement is important in all newly diagnosed DLBCL. However, since FISH analysis is not routinely applicable in all DLBCL new cases, a two-step approach either with a protein expression assessment either with a FISH examination in those cases with a high protein expression, seems today a practical and less expensive sequential approach (Figure 2).

1.1.8. Double expressor, DHL, and THL

Alterations of the *MYC* gene are frequently associated with a *BCL2* rearrangement and/or overexpression (20–35% of cases) [72], while the association with alterations of the *BCL6* gene is less common. The presence of a rearrangement of both the *MYC and BCL2* genes defines the new entities named 'double hit lymphoma (DHL)' or 'triple hit lymphoma (THL)' if there is also a *BCL6* rearrangement. The 2016 WHO classification of lymphoid neoplasms includes all DLBCL with *MYC* and *BCL2* and/or *BCL6* rearrangements in a single category designated as HGBL, with *MYC* and *BCL2* or *BCL6* translocations. DHL and THL are characterized by a particularly aggressive behavior and by a dismal prognosis. Cases with an aberrant expression of MYC and BCL2, without chromosomal alterations, have been defined as 'double expressor lymphoma (DEL)' or 'triple expressor lymphoma (TEL)' if the aberrant expression of BCL6 is also documented [15]. Most HGBL with rearrangements are of GC origin and conversely almost all DEL are of ABC origin.

Johnson et al. [68] studied the frequency of an abnormal MYC and BCL2 expression (29% and 44%, respectively) in a cohort of patients with DLBCL; a concomitant pathological expression of both of them was found in 21% of cases, while a chromosomal translocation of the MYC gene was recorded in 11% of patients. A worse outcome was observed in patients who expressed concomitantly high levels of MYC and BCL2 proteins, and in cases with both genes translocated. When they were treated with the classic R-CHOP schedule, the 5-year OS of the three groups – DEL, DHL, and classic DLBCL – was 36%, 27%, and 71%, respectively (Figure 3). The clinical adverse outcome when both genes are altered pointed to a facilitating role of both of them in the acquisition of a particularly aggressive behavior. It was also clear that patients carrying chromosomal translocations presented a worse outcome compared to patients with only an abnormal expression of BCL2 and MYC.

Green et al. [75] analyzed 193 paraffin-embedded samples of patients with *de novo* DLBCL treated with RCHOP using HIC for MYC, BCL2, CD10, BCL6, and MUM1/interferon regulatory factor 4, and FISH for *MYC* and *BCL2*. They identified 6% of DHL of patients in FISH analysis who presented as expected a very poor OS (p = .002). Furthermore, depending on MYC and BCL2 expression, a double-hit score (DHS) was assigned to all patients with DLBCL. High expression of both MYC and BCL2 protein (DHS2) was significantly associated with lower CR rate (p = .004), shorter OS (p = .001), and shorter PFS (p = .001).

The clinical impact of the *BCL6* rearrangement in DHL is more difficult to define because of the low incidence of this combined alteration. Aukema et al. [76] reported that *MYC/BCL6* DHL maintains a similar clinical outcome as that of patients with *MYC/BCL2* DHL, with an increasing trend in older age compared to *MYC/BCL2* DHL. In contrast with the previous study, a more recent analysis of Ye et al. [77] showed that only DHLs with *MYC* and *BCL2* rearrangement had a significant inferior outcome while the concurrent *MYC/BCL6* rearrangements did not correlate with poorer OS and PFS, similar results were obtained by Copie-Bergman and colleagues [78].

It is still uncertain how to treat these pathologic entities within DLBCL because of the few prospective data. Oki et al. [79] reported that R-EPOCH (rituximab, etoposide, prednisone, vincristine, cyclophosphamide, doxorubicin) versus R-CHOP was associated with a longer EFS. In the largest study of

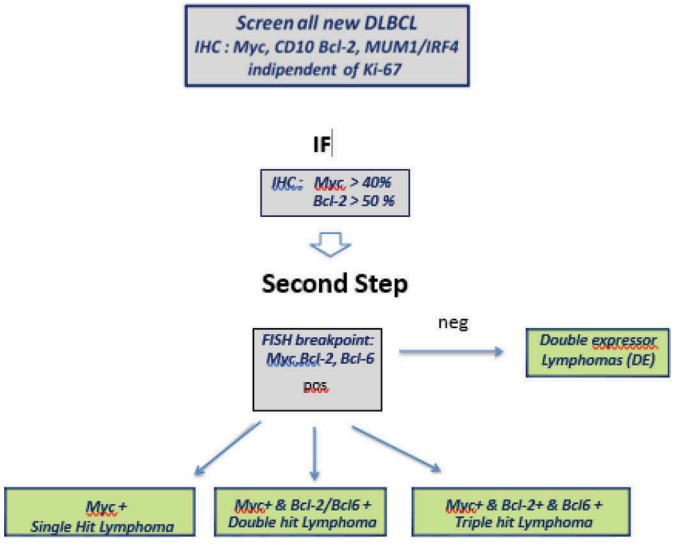

Figure 2. Algorithm for the identification of double hit lymphoma (DHL) and double expressor lymphomas (DEL) if FISH is not applicable for every new diagnosis of DLBCL. The first step evaluates the protein expression of MYC, Bcl2 and Bcl6 by IHC, based on the results only selected cases will be evaluated by FISH analysis.

DLBCL - diffuse large B cell lymphoma; IHC – immunohistochemistry.

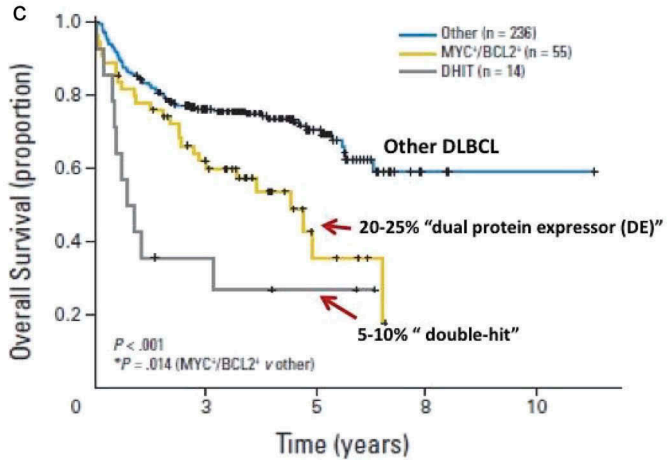

Figure 3. Overall survival of patients with diffuse large B cell lymphoma (DLBCL) according MYC and BCL2 translocation (double hit lymphoma) or MYC and BCL2 protein expression (double expressor, DE). [From Johnson NA et al. J Clin Oncol 2012; 30(28):3452–3459, modified].

patients with DHL, Petrich et al. [80] found that more intensive induction treatment such as DA-EPOCH-R, R-HyperCVAD, or R-CODOX-M/IVAC were associated with a better PFS when compared with the standard R-CHOP ($p = 0.001$). The OS was significantly better in the intensive induction treatment group only after adjusting for adverse risk factors. They also did not find a significant improvement in PFS with consolidative ASCT in first CR. In a recent systematic review and meta-analysis of 394 DHL patients treated with R-CHOP or different alternative strategies as DA-EPOCH-R or high intensity chemotherapy (like R-HyperCVAD or R-CODOX-M/R-IVAC), it was shown that DA-EPOCH-R as upfront therapy can reduce the risk of progression compared to R-CHOP and to other treatments, but the OS was not significantly different among the different schemes [81]. For these reasons, DA-EPOCH-R is under investigation in a prospective study for DHL.

1.2. Toward molecular-driven treatment strategies

Currently, a subtype-specific treatment for patients with DLBCL could become a reality in view of the improved molecular technologies and of the development of new targeted drugs.

IHC and GEP techniques that permit a real-time evaluation of the COO on FFPET tissues are now available; the possibility of recognizing rapidly the molecular COO subtype is thus easier and potentially feasible in the clinical practice. Furthermore, the improved knowledge of the signaling pathways, genetic mutations, and biomarkers operational in lymphomagenesis has led to the expansion of new, biologically based therapies with a differential activity in specific DLBCL subtypes, particularly ABC-DLBCL.

The rationale of the greater activity of new drugs in ABC-DLBCL resides in the mechanism of lymphomagenesis of this subtype that owes its name from the post-GCBs arrested along the plasmacytic differentiation pathway. ABC-DLBCL has a gene expression signature that is largely similar to that of peripheral blood B cells, including the expression of genes that define the end stage memory B cells or plasma cells, such as the transcription factors interferon response factor 4 (IRF4) and X-box binding protein 1 (XBP1) [82]. The constitutive expression of IRF4, which is normally expressed during lymphocyte activation, has been shown to be an important part of the proliferative stimulation, as well as the *XBP1* gene [83]; recently, mutations of the *MYD88* gene (which is known to be mutated in Waldestrom's macroglobulinemia) have been reported to have a role in the activation of the IRF4 pathway [84]. The most important hallmark of ABC-DLBCL is the upregulation of BCR signaling by the NF-kB proliferative pathway [84–86]. The important role of the alteration of the NF-kB family is underlined by the evidence that it is able to induce resistance to apoptosis [87]. The *in vitro* inhibition of the NF-kB pathway using inhibitors of NF-kB has proven to be particularly cytotoxic in ABC-DLBCL but not in GCB-DLBCL [85]. In addition to the NF-kB pathway, other mutations play a role in ABC-DLBCL pathogenesis, like the activation of the specific B-cell surface antigens CD79A and CD79B [82].

On the other hand, an important hallmark of the GCB-DLBCL subtype is the absence or low expression of the NF-κB pathway: it has been demonstrated that GCB-DLBCL survival is not influenced by stimulation through NF-κB [82]. This observation has important clinical implications because drugs which downmodulate the activity of the NF-κB pathway are likely not to have a role in this setting. Interestingly, activating mutations of the histone methyltransferase EZH2 gene (enhancer of zeste homolog 2) have been demonstrated to play an important role in the process of GCB-DLBCL differentiation [88].

Considering the above, a number of clinical trials have investigated novel therapeutic agents targeting the BCR pathway or its downstream constituents in all DLBCLs. These comprise mainly immunomodulatory drugs (IMiDs), proteasome inhibitors and BCR signaling pathway inhibitors (Table 1).

1.2.1. Lenalidomide

Lenalidomide, as other IMiDs, has antineoplastic effects that include direct antineoplastic activity, immunologic effects mediated by inhibition of tumor cell proliferation, angiogenesis, and stimulation of cytotoxicity mediated by immune cells of the tumor microenvironment, including B, T, NK, and dendritic cells [89]. Phase II studies have demonstrated the efficacy of lenalidomide as single agent in relapsed/refractory NHL; in particular, improved response rates and PFS have been observed in ABC-DLBCL [90]. The activity of lenalidomide has been confirmed also in relapsed/refractory DLBCL as single agent or in combination with rituximab. Despite the small sample size (only 23 patients), Zinzani et al. [91] published data that indicate the potential efficacy of lenalidomide and rituximab followed by lenalidomide maintenance in relapsed/refractory elderly DLBCL patients with a CR rate of 26%, a median duration of CR of 5 years (range 30–78 months) and a DFS of 75% at 6 years. The greatest efficacy of lenalidomide in ABC-DLBCL depends on direct targeting of IRF4, leading to the downregulation of the NF-kB pathway activity and enhancement of the interferon pathway [92]. Recently, the results of two phase II trials of lenalidomide combined with

Table 1. Novel drugs with a focus on their biological targets and their activities on GC and ABC DLBCL.

Drug	Target	DLBCL ABC	DLBCL GC
Lenalidomide	Microenvironment	+++	–
Bortezomib	NF-KB	+++	–
Venetoclax	Pro-apoptotic Bcl-2 family	+	++
Ibrutinib	BTK	+++	+
Alisertib	AURORA kinase	++	–
BAY1238090, 0610, OTX015, and JQ1	BET bromodomain	Preclinical data	Preclinical data

NF-KB: nuclear factor kappa-light-chain-enhancer of activated B cells; BTK: Bruton's tyrosine kinase; GCB: germinal center B cell; ABC: activated B cell; DLBCL: diffuse large B cell lymphoma.

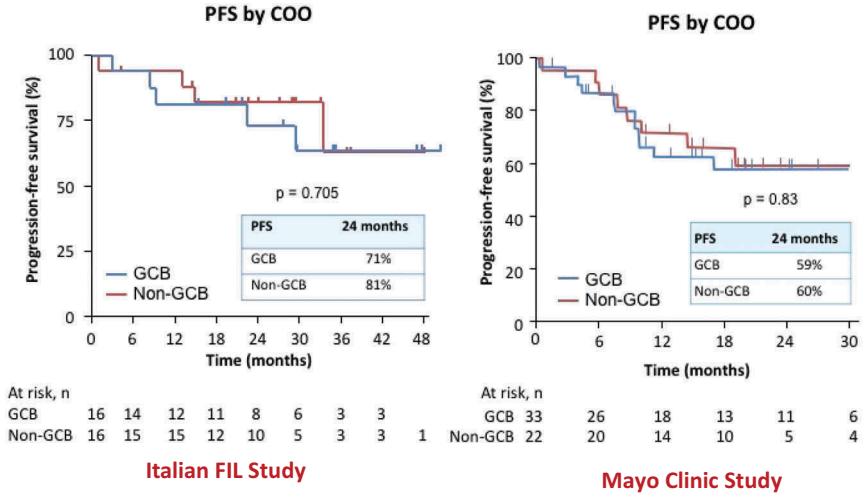

Figure 4. 2-years PFS in GCB-DLBCL and non-GCB DLBCL for patients treated R2-CHOP in the Italian.

FIL (Federazione Italiana Linfomi) and Mayo clinic studies; PFS - progression-free survival; GCB-DLBCL - germinal center-diffuse large B cell lymphoma; R2 - rituximab + lenalidomide; CHOP - cyclophosphamide, doxorubicin, vincristine, prednisone.

R-CHOP in untreated DLBCL have been reported. The first study by the Italian group (REAL07 trial) demonstrated an overall response rate (ORR) of 92% (CR 86%, PR 6%) and 2-year PFS and OS of 80% (95% CI 64–89) and 92% (95% CI 79–97), respectively [93]. In the second study, the Mayo Clinic also reported an ORR of 98% (59/60 patients) with 80% of CR (48/60), and EFS and OS rates at 2 years of 59% (95% CI 48–74) and 78% (95% CI 68–90), respectively. Significantly, the study showed no difference in 2-year PFS or OS for lenalidomide plus R-CHOP in the ABC and GCB subtypes (60% vs. 59%, $p = 0.89$ and 83% vs. 75%, $p = 0.61$); the addition of lenalidomide to the standard treatment of DLBCL (R-CHOP) could overcome the negative prognostic effect of the ABC phenotype [94] (Figure 4). Based on these data, a randomized, double blind, phase III trial (ROBUST; NCT02285062) comparing lenalidomide plus R-CHOP versus placebo in untreated ABC-DLBCL according to GEP analysis on FFPET is now ongoing and actively recruiting. The primary endpoint of this study is PFS [95] (Figure 5).

1.2.2. Bortezomib

Proteasome inhibitors, such as bortezomib, have a key role in the suppression of the NFkB pathway that is constitutively activated in ABC-DLBCL, as discussed. Bortezomib was one of the first drugs investigated that demonstrated favorable results in increasing the activity of chemotherapy in relapsed/refractory DLBCL based on the molecular subtype, as showed by Dunleavy et al. in multicenter trial [96]. However, only 49 patients were enrolled in this trial from 3 centers and it would be ideal designing a randomized trial of DA-EPOCH plus or minus bortezomib with stratification by GCB and ABC DLBCL subtypes to get more significant results. Ruan et al. [97] in a phase II single arm study evaluated the combination of bortezomib with R-CHOP in 40 previously untreated DLBCL and mantle cell lymphoma, and showed that unlike in DLBCL treated

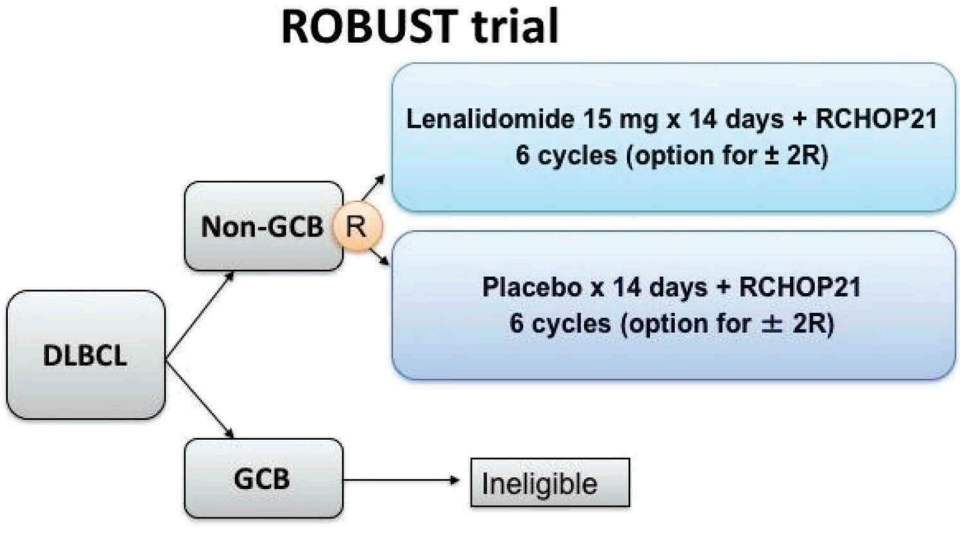

Figure 5. Robust trial: study design. In this trial, the determination of the cell of origin was performed with Nanostring technique.

DLBCL - diffuse large B cell lymphoma; GCB - germinal center B cell; R - rituximab; CHOP - cyclophosphamide, doxorubicin, vincristine, prednisone.

with chemoimmunotherapy alone, patients with ABC-DLBCL had a similar outcome of GCB-DLBCL. Considering this result, the combination of Bortezomib R-CHOP has been evaluated in two phase II randomized trials: LYM-2034 trial and PYRAMID trial. In the first trial, bortezomib plus R-CAP or/and plus R-CHOP was compared to R-CHOP alone in patients with ABC-DLBCL, failing to show an improved efficacy of bortezomib plus immunochemotherapy in terms of ORR and PFS.

In the PYRAMID trial, there were also no significant differences between bortezomib combined with R-CHOP and R-CHOP in 2-year PFS (82% vs. 78%, $p = 0.76$) or OS (93% vs. 88%, $p = 0.76$) [98,99]. Meanwhile, a randomized, double-blind, phase III study (REMoDL-B trial) has completed accrual. The aims of this study were to compare R-CHOP plus bortezomib to R-CHOP alone in DLBCL as front-line therapy determining the genetic subtype by central GEP assay. Based on the preliminary analysis, no difference in terms of ORR and CR was observed between the ABC and GCB molecular subtypes. The final results on 2-year PFS, primary endpoint of the study, are still pending following a 30-month follow-up analysis [100]. Overall, these results seem to prove that bortezomib did not enhance the outcome in ABC–DLBCL; however, we can wait for the final results of the REMoDL-B trial regarding the 2-year PFS to confirm this hypothesis.

1.2.3. Ibrutinib

Another important mechanism in the constitutive activation of the NFkB pathway through the BCR is modulated by the Bruton tyrosine kinase (BTK) and several specific BTK inhibitors have been developed. Ibrutinib is a BTK inhibitor that covalently blocks this TK and is orally bioavailable. Recently, in a phase I/II multicenter study that involved 80 patients with relapsed or refractory DLBCL, ibrutinib as single agent produced complete or partial responses in 37% (14/38) of patients with ABC-DLBCL, but only in 5% (1/20) of GCB-DLBCL patients ($p = 0.0106$). One of the interesting components of this study was the mutational status analysis of the tumors. ABC tumors with CD79a/CD79b (BCR subunits) and MYD88 mutations showed a high response rate, a result that is in line with the cross communication *in vitro* between BCR and MYD88 pathways. However, the highest number of responses was observed in ABC-DLBCL patients lacking BCR mutations (9/29; 31%), suggesting that the oncogenic BCR signaling might be initiated by nongenetic mechanisms [101].

In a phase Ib study of ibrutinib plus R-CHOP for treatment-naïve DLBCL patients, Younes et al. [102] demonstrated that the combination with ibrutinib was well tolerated and that 18 patients who received the recommended phase 2 dose witnessed an overall response. On the basis of these data, a randomized, double-blind, phase III trial (PHOENIX, NCT01855750) comparing R-CHOP versus R-CHOP plus ibrutinib in untreated ABC-DLBCL has completed recruitment. The primary endpoint of the study is the EFS at 7 years, and the preliminary results are pending (Figure 6).

1.2.4. New agents

Most agents active in ABC-DLBCL have little efficacy in the GCB subtype. Patients with GCB-DLBCL have a better clinical outcome than those with ABC-DLBCL, but a relapse after induction therapy is associated with a poor outcome regardless of the COO. Moreover, DHL have a GCB phenotype in the majority of cases. Mostly in DHLs and GCB-DLBCLs new agents may be used according to two strategies: (1) to modulate the transcription of MYC, BCL2, or BCL6; (2) to target the MYC,

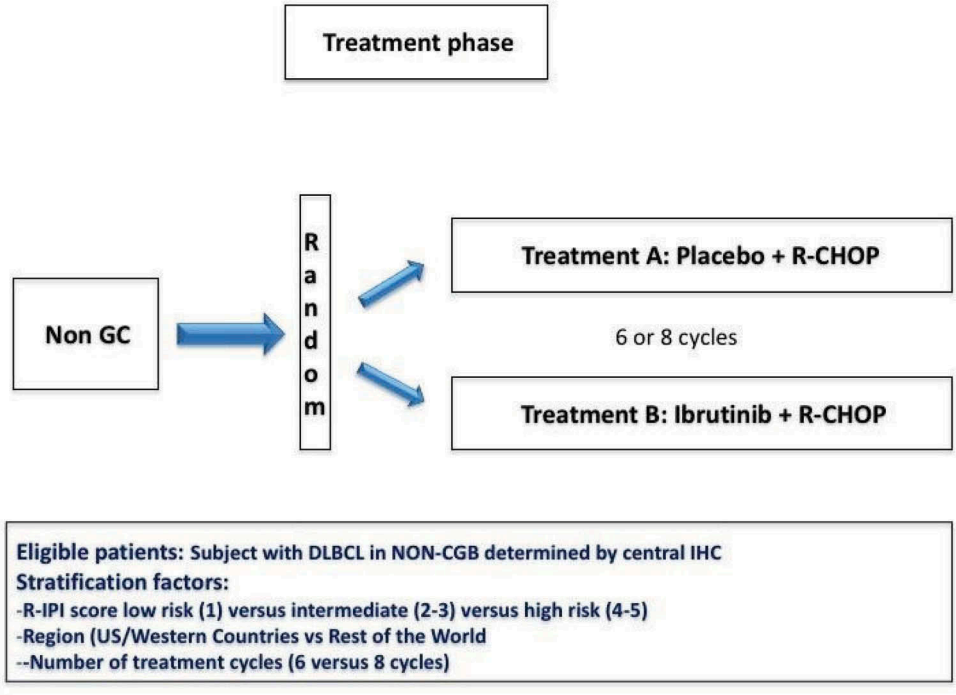

Figure 6. Phoenix trial: study design. In this trial, the determination of the cell of origin was performed with Immunohistochemistry alghoritm.
GCB- germinal center B cell; IHC- immunohistochemistry; R-IPI- revised international prognostic index

BCL2, or BCL6 proteins. For the first strategy, BET bromodomain inhibitors are currently in preclinical development (BAY12380907, CPI-0610, OTX015, and JQ1) [103] and the first reports look promising. JQ1 is a small-molecule inhibitor of BET bromodomain that has shown *in vitro* activity by inhibiting the binding of the BRD4 co-activator (BET family) to chromatin and suppressing MYC-driven transcription. Studies in preclinical mouse models have demonstrated that JQ1 causes cell death, and cell-cycle arrest in DLBCL cells of both GCB and ABC subtypes [104]. Preliminary results of a phase I study with OTX015, another BET bromodomain inhibitor, have demonstrated a good activity and a good safety profile. OTX015 *in vitro* is able to target the NF-κB/TLR/JAK/STAT signaling pathways, genes modulated by MYCC and E2F1, and its activity *in vitro* results synergistic with mTOR and BTK inhibitors [105].

The BCL2 family and the MYC protein also represent a target for new agents as navitoclax and venetoclax (BCL2-inhibitors), and a MYC-targeting aurora A kinase inhibitor (alisertib). Navitoclax (ABT263) showed antilymphoma activity in a phase I trial, but this was associated with a high hematologic toxicity with thrombocytopenia due to BCL-xL inhibition [106]. Venetoclax (ABT-199) is another BCL2 inhibitor engineered to remove the BCL-xL inhibition and thus to reduce the risk of thrombocytopenia. Venetoclax has shown activity in DHL and THL cell lines. However, in a phase I study in patients with relapsed/refractory NHL, the ORR was highest in Waldestrom's macroglobulinemia (4/4) and in mantle cell lymphoma (21/28, 75%); the activity of venetoclax in DLBCL was present but modest (6/34, 18%), with only four patients obtaining a CR (12%) [107].

Alisertib, an aurora kinase inhibitor, has been combined with rituximab and vincristine in a mouse model with DLBCL coexpressing MYC and BCL2, and a high efficacy in inducing cell death and a potential cure has been reported [108].

2. Expert commentary

The era of treating all patients with DLBCL only with the conventional R-CHOP regimen is getting to an end and the current challenge in DLBCL therapy is to move new promising agents to the front-line treatment (X + R-CHOP) in order to improve the results of R-CHOP. As a first step to improve outcome in DLBCL, it is necessary to identify at diagnosis patients with poor prognostic features. Since GEP is expensive and not readily available to all laboratories, and the IHC algorithms lead to a low reproducibility between different laboratories, the 2008 WHO classification considered as provisional the subclassification of DLBCL-NOS. Since then we have been witnessing a better understanding of the genetic mechanisms underlying the disease. The prognostic value of the COO has been conclusively confirmed, with the ABC subtype having a significantly inferior 3-year PFS (40% vs. 75%; $p = < .001$) in patients treated with R-CHOP. In addition, we have unraveled the very different oncogenic pathways that are important prognostically and could have relevant therapeutic implications. In the revised 2016 WHO classification, it is required that the two genetic subtypes of DLBCL – GCB/ABC – are identified at diagnosis. The IHC is accepted instead of GEP and the Hans algorithm is still the most utilized even though new methods as Lymph2Cx using paraffin-embedded tissue will be accessible to most laboratories. In fact, the 20-gene assay using the Nanostring technology gives concordant results with GEP, is reproducible between laboratories, and may become a robust alternative to the current IHC-based algorithm.

Additionally, it is necessary to recognize cases of DLBCL with MYC and BCL2 and/or BCL6 rearrangements, DHLs or THLs now designated as HGBL by the revised 2016 WHO classification, distinguishing those from cases without rearrangements with high MYC and BCL2 expression, DE lymphomas. This is a highly critical point because DHL should not be treated with R-CHOP but they should receive intermediate or high intensity schemes of chemotherapy as reported in many retrospective studies.

Because of the lack of prospective data, we need new prospective trials to better investigate how to treat DHL. Contrary, the COO is under investigation in several first-line randomized phase II and III trials evaluating the efficacy of R-CHOP combined with the new agents with the aim of improving the PFS of those cases of DLBCL-NOS with poor outcome identified as ABC-DLBCL.

The CC-5013-DLC002 (ROBUST trial) is an ongoing randomized study which is investigating the addition of lenalidomide to R-CHOP in untreated ABC type DLBCL, using the Nanostring technology. The other ongoing trials are the Phoenix trial that has evaluated the combination of ibrutinib with R-CHOP in ABC-DLBCL and the REMoDL-B study that has investigated the addition of bortezomib to R-CHOP. These studies have completed their accrual and we are waiting for the preliminary results. These studies could also give us information about the treatment of DEL because most patients express an ABC phenotype reflecting a constitutive NF-kB activation.

3. Five-year view

In the next few years, a greater availability of a network of sophisticated laboratory methodologies aimed at a better understanding of the biology of DLBCL in the real life day-to-day clinical practice will be required. The Nanostring technology may become a promising alternative to IHC analysis; the test is not expensive, may prove reproducible and capable of creating an added value at defining the COO in only a few days. Knowing the COO at the onset of the disease will be important to decide the specific treatment if the results of the current studies will confirm that X-RCHOP is better than R-CHOP in a given subgroup of patients, leading to a personalized upfront therapy truly guided by the molecular characteristics of the disease.

Key issues

- The evaluation of the cell of origin identifies two major subtypes of DLBCL, germinal center B-cell like (GCB) and activated B-cell (ABC) lymphoma which are associated with a different prognostic likelihood. The IHC test remains a valid

routine clinical test to identify the COO instead of GEP, until new methodologies – as Nanostring – will be validated.

- To date, the IPI score remains a robust prognostic index, but it does not address the underlying biologic heterogeneity of DLBCL.
- The understanding of the key signalling and regulatory pathways of DLBCL have identified new biologic prognostic factors and new targeted agents have been developed.
- DHL and THL are new pathologic entities defined as HGBL with rearrangements of MYC and BCL2 and/or BCL6 with an aggressive presentation and a very poor outcome following R-CHOP therapy. Intensified strategies, for example Burkitt's like schemes, should be considered for these subtypes.
- DEL cases do not have genetic alterations of MYC and BCL2 but present a double protein overexpression which is associated with a lower CR rate and shorter OS than DLBCL, but have a better outcome than DHL. New combined strategies with biologic agents could improve their outcome.
- New agents that inhibit the NFkB pathway seem to be more effective in ABC-DLBCL because of the constitutive activation of the NFkB-pathway in this subtype.
- The results of the current trials testing the combination of R-CHOP with novel agents (lenalidomide, bortezomib, ibrutinib) – X + R-CHOP – could help to overcome the adverse prognosis of ABC-DLBCL

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