Review Article

Primary diffuse large B-cell lymphoma of the central nervous system: molecular and biological features of neoplastic cells

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Abstract: Central nervous system diffuse large B-cell lymphoma (CNS-DLBCL), formerly named primary CNS lymphoma (PCNSL), is an extranodal aggressive lymphoma manifesting solely in the brain, spinal cord, leptomeninges or in the eye and represents the vast majority of CNS-lymphoma cases. The vast majority of CNS-DLBCL belong to the activated B-cell like DLBCL phenotype, and more precisely to the genetically defined MCD/C5 clusters. Indeed, CNS-DLBCL cells typically present an active B-cell receptor (BCR), Toll-like receptor (TLR) and NF-κB signaling (*CD79B*, *MYD88 L265P*, *PIM1* mutations), alongside with a block of the terminal B-cell differentiation (*TBL1XR1* and/or *PRDM1* mutations), a cell cycle deregulation (*CDKN2A/B* deletions), the capacity of avoiding apoptosis (*BCL2* gains) and escape the immune surveillance (mutations or deletions of *HLA-A*, *HLA-B*, *HLA-C* and *CD58*). The presence of recurrent DNA mutations can be used to track CNS-DLBCL cells in the cerebrospinal fluid (CSF) and in the plasma. CNS-DLBCL shows a generally lower frequency of *TP53* mutations. In agreement with the activated B-cell like (ABC) phenotype, chromosomal translocations affecting *MYC* and *BCL2* genes are uncommon, although the two proteins are often overexpressed by the tumor cells. Differently from *MYC* and *BCL2*, *BCL6* is recurrently translocated in CNS-DLBCL. The peculiar clinical, genetic, and biologic features of CNS-DLBCL both require and allow the design of clinical trials specifically designed for this patients' population. The current manuscript summarizes the current knowledge on the molecular and biological features of CNS-DLBCL, discussing the potential therapeutic and diagnostic implications.

Keywords: Central nervous system (CNS); brain; NF-κB; targeted therapy; liquid biopsy

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Introduction

Central nervous system diffuse large B-cell lymphoma (CNS-DLBCL), formerly named primary CNS lymphoma (PCNSL), is an extranodal aggressive lymphoma manifesting solely in the brain, spinal cord, leptomeninges or in the eye and represents the vast majority of CNS-lymphoma cases (1,2). Since other articles published on the same special issue describe the epidemiology, the pathology, the clinical and therapeutic approaches for CNS-DLBCL, as well as of secondary central nervous system involvement, here we will focus on the genetics and the biology of CNS-DLBCL cells. Remarkably, the main patho-mechanisms for CNS-DLBCL had already been reported before 2012

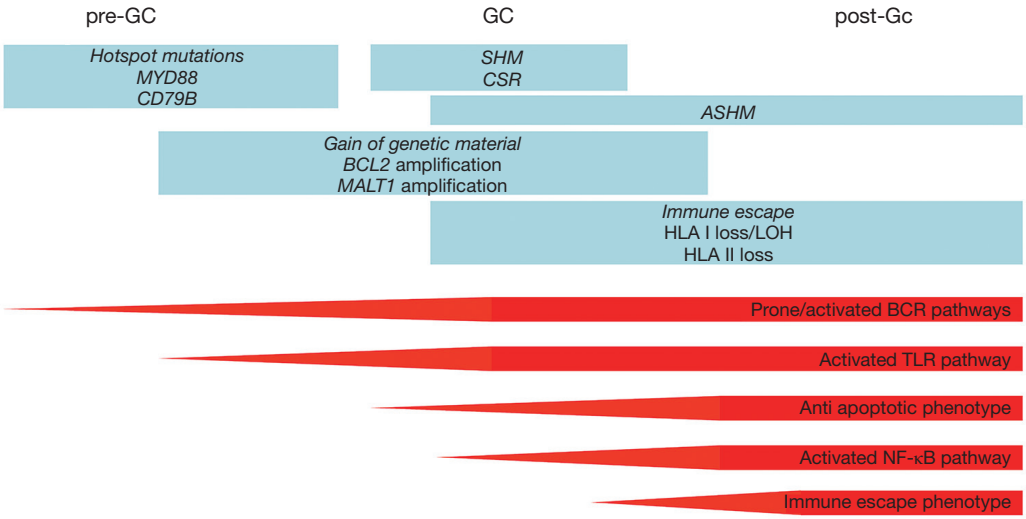

Figure 1 Tumor cell differentiation. Depicted is the timescale of tumor cell differentiation in the pathogenesis of PCNSL and where this alteration could impact in the pathogenesis. *MYD88* and *CD79B* mutations are most likely are acquired before entering a GC reaction, but the selective force during the GC reaction positively selected the precursor of the tumor cell. Most important as pathogenetic mechanism is the faulty GC reaction, during which the precursor cell accumulated mutations and expands, in contrast to the physiological GC reaction, the self-polyreactivity of the B cell receptor. There is no single one event, which determines the development of PCNSL. It is important to understand that all different time points and acquired genetic changes cumulate synergistically in the activation of different pathways and ultimately in the manifestation of PCNSL. PCNSL, primary central nervous system lymphoma; GC, germinal center.

and were subsequently confirmed by numerus publications from different groups (*Figure 1*). Epstein-Barr virus positive CNS-DLBCL show a totally different pathogenesis (3), and we will omit this type of CNS-lymphoma from this review article.

CNS-DLBCL express non-switched self-reactive somatically mutated immunoglobulins

Tumor cells of CNS-DLBCL present immunoglobulin (Ig) heavy and light chain genes with somatic mutations, compatible with their origin from mature B-cells that have encountered antigen and have undergone T-cell dependent somatic maturation (4-12). IGHV4 family genes are frequently rearranged, with a preference for the IGHV4-34 gene segment (4,5,7-9,11).

The tumor cells do not show a successful class switch, as such, they express neither IgA, nor IgE, nor IgG, but only IgM and IgD (13).

The immunoglobulins expressed by CNS-DLBCL cells do not react against self-antigens commonly involved in autoimmune disorders, but they do bind self-proteins, including proteins expressed in the central nervous system, such as GRINL1A, ADAP2 and BAIAP2 (9), or N-hyperglycosylated SAMD14 and neurabin-I (11).

In agreement with the mutated immunoglobulin heavy chain genes, CNS-DLBCL cells also present aberrant somatic hypermutation (ASHM) that affect a series of genes, such as *BTG2*, *HIST1H1E*, *KLHL14*, *MYC*, *PAX5*, *PIM1*, *RHOH*, or *SUSD2* (10,14-16).

CNS-DLBCL have an activated B-cell like (ABC) DLBCL phenotype

DLBCL represents an heterogenous group of diseases (17-20). Studies performed in systemic DLBCL have first identified two main subgroups, the germinal center B-cell like (GCB) and the ABC DLBCL, based on their expression profiles suggestive of their potential cell of origin (COO) (21-24). GCB and ABC DLBCL have differences in genetics and biologic features and, importantly, in the clinical outcome when patients are treated with the standard R-CHOP treatment (18,24). The vast majority of CNS-DLBCL are constituted by non-GCB DLBCL. This has been reproducibly demonstrated in many studies, largely performed using immunohistochemistry (15,25-42), but

also via gene expression profiling (33,43-46).

Differently from what observed in systemic DLBCL, the prognostic impact of the COO in CNS-DLBCL seems to be minimal, with the vast majority of studies not finding any difference in the clinical outcome between patients with ABC or GCB CNS-DLBCL (31-43). Fukumura *et al.* reported an inferior progression-free survival (PFS) for non-GCB than GCB cases in a series of 39 patients (15). Shi *et al.* reported a borderline inferior 3-year overall survival (OS) for ABC than GCB-PCNS DLBCL when considering 77 patients treated half with methotrexate-based scheme and half not, but the difference was not maintained when they analyzed the outcome based on treatment modality (29).

CNS-DLBCL cells belong to a specific ABC DLBCL cluster

In the last few years, ABC and GCB DLBCL have been further subclassified in additional sub-groups that share more homogenous genetic lesions (47-50). In particular, a specific ABC DLBCL subset has been recurrently identified, although named in different ways: C5 (47), MCD (48,49) or MYD88 cluster (50). These lymphomas are characterized by the presence of genetic events that activate the B-cell receptor (BCR), the Toll-like receptor (TLR) and NF-κB signaling (often concomitant *CD79B* mutations and *MYD88* L265P mutation; *PIM1* mutations), block the terminal B-cell differentiations (*TBL1XR1* and/or *PRDM1* mutations), deregulate the cell cycle (*CDKN2A/B* deletions), allow the immune escape (mutations or deletions of HLA-A, HLA-B, HLA-C and *CD58*), or protect from apoptosis (*BCL2* gains), plus additional mutations in *KLHL14* or *ETV6* (47-50).

Importantly, CNS-DLBCL cells exactly presents a pattern of lesions overlapping with this newly described MCD subtype. Whereas CNS-DLBCL, or more general CNS-lymphoma, refers to a lymphoma manifestation in the CNS, the term CNS-DLBCL should be restricted for CNS-DLBCL of this MCD subtype. Different studies have reported a very high frequency of mutations occurring in *CD79B*, *MYD88* (10,15,16,30,33,51-73), *PIM1* (10,15,30,33,53,56-60,65,71,72) and TBL1XR (56,57,59,67,72), *BCL2* locus gains (25,39,40,58,74,75), inactivation of *CDKN2A* (30,55,58,59,62,69,76-87), *PRDM1* (59,75,81,85-92) and *ETV6* (57,58,93), and genetic lesions leading to immune evasion (15,56,59,78,81,85-87,94).

The BCR/TLR/NF-κB signaling is active in CNS-DLBCL

Multiple mechanisms sustain the constitutive activation of the BCR/TLR/NF-κB signaling. As in the C5/MCD/MYD88 cluster, mutation of *CD79B* and MYD88 usually co-occur. The *MYD88* mutations are mostly represented by the c.794T>C substitution resulting in leucine 265 replaced by proline (L265P). Hotspots are also observed in *CD79B*, with replacement of tyrosine 196 with either asparagine (Y196N), aspartic acid (Y196D), cysteine (Y196C), histidine (Y196H) or serine (Y196S) as the most observed. A lower frequency of MYD88 L265P mutations has been reported in PCSNL from Hispanic versus non-Hispanic patients: 27% (5/18) *vs.* 66% (8/12) (53).

The gene coding for the PIM1 serine/threonine kinase is among the most mutated. PIM1 is known to be involved in lymphomagenesis (95-98). BCL6 transgenic mouse models have shown that *PIM1* cooperates with BCL6, recurrently translocated in CNS-DLBCL (99,100), to give lymphomas (97). Pharmacological and genetic preclinical experiments in cell lines derived from ABC DLBCL, primary mediastinal large B-cell lymphoma and from Hodgkin lymphoma indicate a role for *PIM1* in sustaining the lymphoma cells survival, activating NF-κB and JAK STAT signaling and immune evasion (98,101,102).

Similarly to systemic ABC DLBCL, recurrent gains are observed at 3q12.3 leading to the overexpression of *NFKBIZ* gene coding for the NF-κB co-activator IκB-ζ (58). Functional experiments performed on systemic DLBCL cell lines show that *NFKBIZ* silencing induces apoptosis and reduces cell proliferation and expression levels of IκBα and BCLXL (58). A similar role might be envisioned also for the NF-κB co-activators IκB-α and IκB-ε, coded by *NFKBIA* gene and *NFKBIE*, also recurrently mutated in DLBCL (103,104).

Kaulen *et al.* have reported mutations in *SLIT2* in 3/6 CNS-DLBCL studied by whole exome sequencing (72). Mutational pattern and functional experiments performed, in the non-lymphoma model HEL293T cells transfected with mutated or wild type SLIT2, suggest that the gene might have a tumor suppressor function, negatively regulating WNT- and NF-κB signaling (72). Its inactivation would contribute to NF-κB signaling in CNS-DLBCL.

TERT (telomerase reverse transcriptase) mutations have also been reported in 16% (16/49) of CNS-DLBCL (105). TERT is involved in the maintenance of telomeres and it

is both an NF-κB target and NF-κB cofactor. Thus, the mutations would represent an additional mechanism to active BCR/NF-κB pathway (105).

PRDM1 gene, coding for the plasma cell master regulator BLIMP1, is mapped at 6q21, a region commonly deleted in CNS-DLBCL, with DNA losses that often encompass also *TNFAIP3*, coding for A20, a negative regulator of NF-κB target, and other potential tumor suppressor genes (59,75,81,85-92,106). A similar pattern with a large genomic aberration potentially deregulating both B-cell terminal differentiation and NF-κB, is also observed in systemic ABC DLBCL.

BAFF (B-cell activating factor of the tumor necrosis factor family) and APRIL (a proliferating inducing ligand) are among the signals that lead to activation of the NF-κB pathway in mature B-cells. In particular, APRIL binds to two receptors, TACI (transmembrane activator and CAML interactor) and BCMA (B-cell maturation antigen), while BAFF binds TACI, BCMA, and also BAFF-R (BAFF-receptor).

CNS-DLBCL cells express all three receptors (107,108). The production of the ligands by astrocytes, as shown for BAFF, or, possibly by the neoplastic cells themselves, would contribute to the survival of tumor cells (107-109). Also, the soluble forms of BCMA and TACI (110,111) have been reported elevated in the cerebrospinal fluid (CSF), but not in the serum of CNS-DLBCL patients (110). Similarly, both APRIL (112) and BAFF (111) can be detected in the CSF of CNS-DLBCL patients.

Muta *et al.* have suggested that another mechanism that might contribute to NF-κB activation in CNS-DLBCL cells is the ghrelin/GHS-R (growth hormone secretagogue receptor) axis (113). CNS-DLBCL cells express both proteins (113) and, in glioma cells, ghrelin activates NF-κB signaling and induces migration of the cells (114).

Finally, the activation of the NF-κB and JAK/STAT signaling, characteristic of the ABC phenotype, are reflected by the presence of secreted factors such as interleukin 10 (IL-10) and of CXC chemokine ligand 13 (CXCL13), which can be detected in CSF, representing a potential diagnostic biomarker and tool to follow patients during therapy (115-130).

CDKN2A is frequently inactivated in CNS-DLBCL

CDKN2A inactivation is very common in CNS-DLBCL (30,55,58,59,62,69,76-87,131), again reflecting its similarities with systemic ABC DLBCL C5/MCD/MYD88 cluster (47-50). Braggio *et al.* has reported over 80% of cases with *CDKN2A* inactivation by mutations and/or deletions at 9p21.3 (56). Nayyar *et al.* showed biallelic loss in 44% of 6 CNS-DLBCL cases (69). Cobbers *et al.*, reported homozygous deletion in 8/20, plus heterozygous loss in additional two cases (77). A mechanism of *CDKN2A* inactivation is also mediated by promoter hypermethylation (77,131). It is worth of mentioning that *CDKN2A* losses are matched with a high level of genomic complexity, including deletions at 3p14.2 affecting the tumor suppressor *FHIT* (58).

TBL1XR1 mutations and reprogramming the differentiation

Mutations in *TBL1XR1* were first described in CNS-DLBCL by Gonzalez-Aguilar *et al.* (59), and were considered to play a regulatory role in the NF-kB pathway. The underling mechanism is much more complex, because *TBL1XR1* mutations will impair plasma cell differentiation. Thereby shifting the B-cell differentiation to non-class-switched IgM memory B-cells with an aberrant cyclic reentry to new germinal centers (132,133), which reflects the phenotype of the tumor cells in CNS-DLBCL (13,44).

Epigenetic regulators are only marginally affected

Mutations in genes coding for epigenetic regulators, in particular *EZH2*, *CREBBP*, *EP300*, are very uncommonly mutated in CNS-DLBCL (15,30,34,57,72,134), and only KMT2D seems to be mutated in 10-40% of the cases (15,30,33,71). The pattern is in agreement with what reported in systemic ABC DLBCL belonging to the C5/MCD/MYD88 cluster (47-50).

TP53 can be mutated in CNS-DLBCL

TP53 mutations have been reported at very different frequencies across studies (15,26,33,71,76,77,82,85,90). Perhaps reflecting the ABC DLBCL C5/MCD/MYD88 cluster (47-50), CNS-DLBCL shows a generally lower frequency of TP53 mutations than considering systemic DLBCL as a whole. Lauw *et al.* have suggested a higher prevalence of *TP53* mutations and lower percentage of mutations in genes involved in BCR/TLR/ NF-κB signaling (e.g. *MYD88*, *CARD11*, *CD79B*, *PIM1*) in pediatric than adult CNS-DLBCL (135).

MYC, BCL2 and BCL6 rearrangements

In agreement with the ABC phenotype, chromosomal translocations affecting *MYC* and *BCL2* gene are uncommon (25,35,36,39,40,74,91,136-139). However, CNS-DLBCL often overexpress MYC and BCL2 proteins (29,31,32,36,38-40,91,138), and gains or amplifications of the genomic loci are detected in 10–30% (25,36,39) and 20–40% (25,39,40,58,75), respectively (74).

Differently from *MYC* and *BCL2*, *BCL6* is recurrently translocated in CNS-DLBCL with frequencies ranging from 17% to 44% (25,34,39,40,58,74,91,99,100,137-139), perhaps more commonly than what seen in the systemic ABC DLBCL C5/MCD/MYD88 cluster (47-50).

The ETS transcription factor ETV6 is frequently inactivated

The ETS transcription factor ETV6 appears inactivated by multiple mechanisms in CNS-DLBCL (57,58,93) as well as in systemic C5/MCD/MYD88 cluster (47-50). The gene is targeted by mutations in 16% (6/37) (57) and deletions in exons 2 or exons 2–5 in 13% (3/24) (58). Finally, the gene is translocated in the ETV6-IGH fusions in 18% (13/72) of the cases, with breakpoints leading to a truncated ETV6, lacking exons 1–2 (93). Albeit ETV6 is known to be involved in B-cell development, the contribution to CNS-DLBCL pathogenesis of its inactivation is still to be defined.

Different potential mechanism of immune evasion

Immune escape by loss of expression of MHC class I is a fundamental mechanism in DLBCL (140). Genetic events reported in systemic DLBCL and indeed enriched in the C5/MCD/MYD88 cluster (47-50,141) as mechanism to evade the immune system are common in CNS-DLBCL: B2M, *CD58*, CIITA are often targeted by somatic mutations, deletions or genomic rearrangements (15,56,58,59,78,81,85-87,94).

Integrating CNV and WES Braggio *et al.* reported inactivation of HLA genes by somatic mutations in 50% and B2M inactivation in approximately 10% of the cases (56). In a series of 19 CNS-DLBCL cases, Schwindt reported DNA losses or uniparental disomy in 74% of the cases at 6p21.32, the region spanning the MHC class I and II encoding genes (78). Fukumura *et al.* reported mutations in *CD58* in 17%, B2M in 10%, CIITA (17%), HLA-C (15%), and focal deletions of HLA locus in one case (15).

The relevance of PDL1/PDL2 in CNS-DLBCL is undefined. Chapuy *et al.* reported chromosomal translocations the PDL1 locus in 3/24 cases (13%) (58). Genomic amplifications of PDL1/PDL2 are uncommon (25,69,71,78,142), and even their effect is uncertain since Chapuy *et al.* but not Villa *et al.* have reported concomitant protein over-expression in cases with genomic gain of the locus (25,58).

Finally, Waldera-Lupa *et al.* have studied CSF proteome in 17 CNS-DLBCL compared to 10 non-tumor samples (143). HLA class II HLA-DRB1 was among the proteins increased in CNS-DLBCL patients, suggesting that shedding of the detected protein could represent a mechanism of immune evasion (143).

miRNAs and CNS-DLBCL

Despite the rarity of the disease, quite a few studies have been published regarding the potential role of miRNAs (144-153). Known oncomirs such the members of the miR-17–92 cluster, in particular miR-17-5p, miR-155, miR-21 miR-196b, have been reported upregulated in CNS-DLBCL, similarly to other lymphoma subtypes (144-146,149-151). Unfortunately, due to the still uncharacterized function of most miRNAs, the results of the studies, reviewed elsewhere (154), are largely preliminary and descriptive.

Takashima *et al.* have presented a prognosis prediction model based on four miRNAs (miR-101, miR-548b, miR-554, and miR-1202), identified profiling 40 CNS-DLBCL focusing on miRNAs that regulate immunity (147).

Interestingly, miR-21, miR-19b, and miR-92a are more expressed in the CSF of patients with CNS-DLBCL than non-malignant brain lesions and might have a biomarker role (146,149-151), as also proposed for the U2 small nuclear RNA fragments (RNU2-1f), also detectable at high levels in the CSF of CNS-DLBCL (155).

Genetic lesions and clinical outcome

There are no clear associations between the presence of any genetic lesion and clinical outcome. This is due to the sample size of the series, the heterogeneity of therapies, the frequency of the lesions, and the different methodologies to define the presence of absence of the genetic event.

Across a series of heterogeneous 54 CNS-DLBCL,

Table 1 Main genetic and biologic features of CNS-DLBCL

Cell of origin	ABC-DLBCL
Genetic subtype	C5/MCD
Immunoglobulin genes rearrangement	Non-switched self-reactive somatically mutated immunoglobulins
Recurrent mutations	*CD79B*, *MYD88*, *ETV6*, *PIM1*, *SLIT2*, *TERT* (BCR/TLR/NF-κB), *HLA-A*, *B2M*, *CD58* (immune escape), *ETV6*, *TBL1XR1*, *PRDM1* (B cell differentiation), *KMT2D* (chromatin remodeling)
Recurrent genomic gains	+18q (*BCL2, MALT1*), +3q (*NFKBIZ*), +18p, +3p, +19q13.42, +19q
Recurrent genomic losses	-6p21.32 (*MHC*), -6q21 (*PRDM1*), -9p21.3 (*CDKN2A*)
Recurrent fusions and translocations	3q27 (*BCL6*)

CNS-DLBCL, central nervous system diffuse large B-cell lymphoma; ABC, activated B-cell like.

Zheng *et al.* reported a worse outcome for the four patients with mutated *CD79B* versus the 12 wild type patients (70). An inferior PFS for mutated *CD79B* cases has been seen also in a series of 57 cases with a mutation frequency of 59% (30) but not in another series of 71 patients (67).

No prognostic impact has been reported for the presence of MYD88 mutations in different series (59,67,70,156).

Deletions at 6q have been associated with inferior outcome in multiple series, also including HD-MTX treated patients (59,89,91,92).

Loss at 6q22, alongside with the presence of *BCL6* translocation, was associated with inferior outcome in a series of 75 cases of CNS-DLBCL (38% treated with HD-MTX) (91).

Other genetic lesions potentially affecting the outcome of CNS-DLBCL patients include *CDKN2A* homozygous losses (59), *BCL6* translocations (91), HLA-C mutations (15).

Munch-Petersen *et al.* studied TP53 in 86 CNS-DLBCL patients. Although the 32 mutated cases (34%) did not show differences in clinical outcome, a worse PFS and OS was seen for the nine patients bearing a TP53 mutation supposed to directly affect the direct protein/DNA contact versus all the other patients treated with combination chemotherapy with or without rituximab (26).

DNA mutations can be used to track CNS-DLBCL cells

The analysis of circulating tumor DNA (ctDNA) represents an important tool in the diagnostic and follow-up settings (130,157-161). As for systemic disease, tumor mutations can be detected in serum- or plasma-derived ctDNA of CNS-DLBCL (51,62,64,161,162). Moreover, the analyzed ctDNA in CSF might be more effective in detecting the presence of tumor cells than flow cytometry or cytology (63,163-166), and could be used to follow the patient during and after treatment (164). In the context of a prospective study, Ferreri *et al.* have detected the *MYD88* L265P mutation in the CSF of 26/36 (72%) newly diagnosed and relapsed CNS-DLBCL patients, respectively (130). The combination of CSF IL-10 levels and *MYD88* L265P appeared to identify disease in the CSF of 21/24 (88%) patients with relapsing CNS-DLBCL (130). At the 2021 Meeting of the American Society of Hematology, Mutter *et al.* reported the analysis of 85 tumor biopsies, 131 plasma samples, and 62 CSF specimens from 92 CNS-DLBCL patients using ultrasensitive sequencing technologies such CAPP-Seq (cancer personalized profiling by deep sequencing) and PhasED-Seq (phased variant enrichment and detection sequencing) (161). Genetic lesions were detected in 78% of plasma samples and in 100% of CSF specimens, and the levels of ctDNA, at baseline and during treatment appeared to predict PFS and OS (161). Moreover, the data suggest that the use of plasma or CSF ctDNA could be implemented in the diagnostic workflow to possibly avoid surgical biopsies for a subset of patients (161).

Genetic and biologic features suggest targeted therapies

The enrichment of specific genetic and biologic features in CNS-DLBCL (*Table 1*) provide the rationale for potential targeted therapies (127,167-174), which have been explored in a series of phase I and II studies (*Table 2*).

CNS-DLBCL are highly enriched of *MYD88 L265P* and *CD79B* mutations that provide the strong rationale for testing BTK inhibitors (94,164,176,178,187). Indeed, in systemic ABC DLBCL, these genetic features have

Table 2 Phase I and II trials exploring the use of targeted agents as single agents and in combination in patients with CNS-DLBCL (based on https://clinicaltrials.gov accessed in June 2021)

Clinical phase	Compound	Mechanism of action	Population (cases)	ORR	CRR	NCT Number	Status	Reference, when available
I	Ibrutinib*	BTK inhibitor, chemotherapy, anti-CD20	R/R (n=9)	89%*	67%*	NCT02315326	Active, not recruiting	(175)
I	Lenalidomide	IMID	R/R (n=8)	88%	12%	NCT01542918	Completed	(123)
I	Ibrutinib in the DA-TEDDI-R regimen^	BTK inhibitor, chemotherapy, anti-CD20	R/R (n=13), 1st line (n=5)	83%	86%	NCT02203526	Recruiting	(176)
I	Ibrutinib, rituximab, lenalidomide	BTK inhibitor, IMID, anti-CD20	R/R PCNSL and SCNSL (n=14)	78%	28%	NCT03703167	Recruiting	(177)
I	Ibrutinib	BTK inhibitor	R/R (n=13)	77%	38%	–	–	(94)
I/II	Copanlisib, ibrutinib	BTK inhibitor, PI3Kα/δ inhibitor	R/R (n=6)	67%	17%	NCT03581942	Recruiting	(164)
II	Ibrutinib	BTK inhibitor	R/R (n=52)	52%	20%	NCT02542514	Active, not recruiting	(178)
II	Temsirolimus	mTOR inhibitor	R/R (n=37)	54%	22%	NCT00942747	Unknown	(179)
I	Pomalidomide, dexamethasone	IMID, steroid	R/R PCNSL or 1st line/RR IOL (n=25)	48%	32%	NCT01722305	Completed	(180)
II	Lenalidomide, rituximab	IMID, anti-CD20	R/R (n=50)	36%$	29%$	NCT01956695	Completed	(181)
II	Buparlisib	Pan-Pi3K inhibitor	R/R PCNSL and SCNSL (n=4)	25%	0%	NCT02301364	Completed	(182)
I	Bimiralisib	mTOR/PI3K inhibitor	R/R	n.a.	n.a.	NCT02669511	Completed	–
I	Rituximab, lenalidomide, and nivolumab	IMID, anti-PD1, anti-CD20	R/R	n.a.	n.a.	NCT03558750	Terminated (low accrual)	–
I	Acalabrutinib, rituximab, durvalumab	BTK inhibitor, anti-PDL1, anti-CD20	R/R	n.a.	n.a.	NCT04688151	Not yet recruiting	–
I	Nivolumab, pomalidomide	Anti-PD1, IMID	R/R	–	–	NCT03798314	Active, not recruiting	–
I	CA-4948	IRAK4 inhibitor	R/R	n.a.	n.a.	NCT03328078	Recruiting	(183)
I	PRT811	PRMT5 inhibitor	R/R	n.a.	n.a.	NCT04089449	Recruiting	–
I	Venetoclax, obinutuzumab	BCL2 inhibitor, anti-CD20	R/R	n.a.	n.a.	NCT04073147	Recruiting	–
I	Pevonedistat, irinotecan, temozolomide	NEDD8 inhibitor, chemotherapy	R/R	n.a.	n.a.	NCT03323034	Recruiting	(184)
II	Pembrolizumab	Anti-PD1	R/R	n.a.	n.a.	NCT02779101	Unknown	–
II	Sintilimab	Anti-PD1	R/R	n.a.	n.a.	NCT04052659	Recruiting	–

Table 2 *(continued)*

Table 2 *(continued)*

Clinical phase	Compound	Mechanism of action	Population (cases)	ORR	CRR	NCT Number	Status	Reference, when available
II	Acalabrutinib	BTK inhibitor	R/R	n.a.	n.a.	NCT04548648	Not yet recruiting	–
II	Camrelizumab	PD1	R/R	n.a.	n.a.	NCT04070040	Recruiting	–
II	IBER (ibrutinib, rituximab, ifosfamide, etoposide) followed by ibrutinib as maintenance	BTK inhibitor, anti-CD20, chemotherapy	R/R	n.a.	n.a.	NCT04066920	Not yet recruiting	–
II	F520	anti-PD1	R/R	n.a.	n.a.	NCT04457869	Not yet recruiting	–
II	Ibrutinib *vs.* lenalidomide in combination with MRE (methotrexate, rituximab, etoposide)	BTK inhibitor, IMID, anti-CD20, chemotherapy	R/R	n.a.	n.a.	NCT04129710	Recruiting	–
II	Abemaciclib	CDK4/6 inhibitor	R/R	n.a.	n.a.	NCT03220646	Recruiting	–
II	Ibrutinib, nivolumab	BTK inhibitor, anti-PD1	R/R	n.a.	n.a.	NCT03770416	Recruiting	(185)
II	ICP-022	BTK inhibitor	R/R	n.a.	n.a.	NCT04438044	Recruiting	–
II	Pembrolizumab	Anti-PD1	R/R	n.a.	n.a.	NCT03255018	Active, not recruiting	–
II	Nivolumab	Anti-PD1	R/R	n.a.	n.a.	NCT02857426	Active, not recruiting	–
II	Bimiralisib	mTOR/PI3K inhibitor	R/R	n.a.	n.a.	NCT03120000	Withdrawn	–
I/II	Pembrolizumab, ibrutinib and rituximab	BTK inhibitor, anti-PD1, anti-CD20	R/R	n.a.	n.a.	NCT04421560	Recruiting	–
I	Acalabrutinib and durvalumab	BTK inhibitor, anti-PDL1	R/R, 1st line	n.a.	n.a.	NCT04462328	Not yet recruiting	–
I	TEDDI-R	BTK inhibitor, chemotherapy, anti-CD20	1st line (n=13)	92%	67%	NCT02203526	Recruiting	(186)
I	Nivolumab	Anti-PD1	1st line	n.a.	n.a.	NCT04022980	Recruiting	–
I/II	R2-MTX with lenalidomide maintenance	IMID, chemotherapy, anti-CD20	1st line	n.a.	n.a.	NCT04120350	Recruiting	–
I/II	Lenalidomide and ibrutinib in association with rituximab-methotrexate procarbazine vincristin (R-MPV)	IMID, BTK inhibitor, chemotherapy, anti-CD20	1st line	n.a.	n.a.	NCT04446962	Recruiting	–
II	Ibrutinib, methotrexate, temozolomide	BTK inhibitor, chemotherapy	1st line	n.a.	n.a.	NCT04514393	Not yet recruiting	–
II	Lenalidomide, rituximab, methotrexate	IMID, anti-CD20, chemotherapy	1st line	n.a.	n.a.	NCT04481815	Recruiting	–

Table 2 *(continued)*

Table 2 *(continued)*

Clinical phase	Compound	Mechanism of action	Population (cases)	ORR	CRR	NCT Number	Status	Reference, when available
II	Nivolumab, maintenance	Anti-PD1	1st line	n.a.	n.a.	NCT04401774	Recruiting	–
II	Rituximab, lenalidomide combined with methotrexate and temozolomide	IMID, anti-CD20, chemotherapy	1st line	n.a.	n.a.	NCT04737889	Recruiting	–
II	Ibrutinib as maintenance	BTK inhibitor	1st line	n.a.	n.a.	NCT02623010	Recruiting	–
II	Lenalidomide, rituximab maintenance	IMID, anti-CD20	1st line	n.a.	n.a.	NCT04627753	Recruiting	–
II	Lenalidomide and nivolumab to the usual treatment	IMID, anti-PD1	1st line	n.a.	n.a.	NCT04609046	Recruiting	–
II	Chidamide, rituximab, methotrexate	HDAC inhibitor, anti-CD20, chemotherapy	1st line	n.a.	n.a.	NCT04516655	Not yet recruiting	–
II	Lenalidomide *vs.* procarbazine as maintenance after HDX-MTX-based regimen	IMID, chemotherapy	1st line	n.a.	n.a.	NCT03495960	Recruiting	–

*, in combination with rituximab and high-dose methotrexate and as maintenance; ^, based on ibrutinib alone given for 14-day prior to cycle 1 of DA-TEDDI-R (temozolomide, etoposide, liposomal doxorubicin, dexamethasone, ibrutinib, and rituximab) with intraventricular cytarabine; $, on the 23 patients that received 8 cycles. CNS-DLBCL, central nervous system diffuse large B-cell lymphoma; ORR, overall response rate; R/R, refractory/relapsed; CRR, complete remission rate; IMID, immunomodulatory drug; n.a., not available.

been associated with higher sensitivity to this therapeutic approach (188). Since CNS-DLBCL are highly enriched of these features, it made sense to test the use of the BTK inhibitor ibrutinib (94,164,176,178,187). Preclinical and clinical data show that ibrutinib passes the blood brain barrier (176,189,190), and the drug has been tested both as single agent (94) and in combination (164,176). Interestingly, the relationship between genetic status and clinical responses is intriguing with responses observed also in patients lacking *MYD88/CD79B* mutations, or in patients with tumors with genetic lesions, such as *CARD11* mutations, expected to cause ibrutinib resistance (164,176,178). For example, in the study reported by Grommes *et al.*, non-responders were enriched of lesions supposed to give resistance to ibrutinib (*CARD11* mutations, *TNFAIP3* inactivation), but, differently from systemic DLBCL (99), there were no complete remissions (CR) in patients with tumors bearing both *MYD88* and *CD79B* mutations (three partial response and one stable disease) (94). Two of three CR cases had *MYD88 L265P* but lacked *CD79B* mutations and one was wild type for both *MYD88* and *CD79B* (94).

In vitro evidence obtained in cell lines derived from the systemic ABC DLBCL C5/MCD/MYD88 cluster showed a strong synergism for the combination of ibrutinib with DNA damaging agents (doxorubicin, etoposide, cytarabine, and mitomycin C) and antagonism when the BTK inhibitor was combined with multiple anti-folates, apparently not supporting a combination with methotrexate-based regimens (176). However, clinically, a similar overall response rate (ORR) has been achieved with ibrutinib combined with both DNA damaging agents (176) and methotrexate-based regimens (164).

It must be mentioned that in the phase I combining ibrutinib with DNA damaging agents in 18 CNS-DLBCL patients, major infections were common: five cases of pulmonary aspergillosis, one case of pulmonary *Pneumocystis jiroveci*, and three pneumonitis of undetermined etiology, two cases of CNS aspergillosis, and one case of enterocolitis (176). Eight of the 18 patients died, three from disease progression, two due to Aspergillus infection during the ibrutinib window, and one due to neutropenic sepsis during chemotherapy (176). Preliminary data from the ongoing follow-up study suggest that the introduction of fungal prophylaxis seems to avoid aspergillosis (186).

Clinical studies are now exploring BTK inhibitors also in combination with additional targeted agents such immunomodulatory drugs (IMiDs), anti-CD20 monoclonal antibodies against CD20 or checkpoint modulators. Among the first 15 patients (11 with CNS-DLBCL, four with secondary CNS lymphoma) enrolled in a phase I study combining ibrutinib, with the IMID lenalidomide and the anti-CD20 monoclonal antibody rituximab, no Aspergillosis infection was observed, with thrombocytopenia, lymphopenia, and rash as the most adverse events (177).

In addition to an active BTK signaling, CNS-DLBCL cells also depend on the PI3K/AKT/mTOR and RAS/MAPK signaling (94,191-193). Active mTOR signaling has been reported in over 70% of 24 CNS-DLBCL cases, using phospho-4E-BP1 (Thr 37/46) and phospho-S6 (Ser235/236) as downstream markers of activity (192). The transcriptome of CNS-DLBCL cases with *CD79B* mutations is enriched of gene expression signatures associated with PI3K and mTOR signaling, while NFKB signatures are under-represented (94). The need of targeting of multiple proteins is suggested also by the preclinical observation that the killing of *CD79B*-mutant CNS-DLBCL patient-derived xenografts is not achieved using ibrutinib or isoform specific PI3K inhibitors as single agents, but only with pan PI3K inhibitors, dual PI3Kα/δ inhibition, or combining low concentrations of the BTK inhibitor with dual PI3Kα/δ or mTOR inhibitors (94). Phase I–II studies performed in the relapsed/refractory (R/R) setting have so far reported only modest single agent activity with the mTOR inhibitor temsirolimus (179) or the pan-PI3K inhibitor buparlisib (182). No data have yet been reported with the dual PI3K/mTOR inhibitor bimiralisib. Albeit based on the very first six patients treated in the escalation phase (164), the combination of ibrutinib and the PI3Kα/δ inhibitor copanlisib seems safe but not associated with much better results, at least in terms of response rate, than what seen with single BTK inhibition. It must also be mentioned that the observed phospho-S6 might be downstream to an additional kinase, PASK, and not mTOR, suggesting different therapeutic targets (193).

The typical MCD phenotype of CNS-DLBCL cells, the role of IMIDS in targeting the IRF4/SPIB axis in systemic MCD DLBCL models as well as the reported synergisms with IMDS and ibrutinib (194) represent the rationale to the clinical studies with lenalidomide or pomalidomide as single agents and in combination (123,177,180,181).

There are a whole lot of additional targets that have not yet been clinically explored in the CNS-DLBCL setting.

Jiménez *et al.* reported preclinical synergism combining

Table 3 On-going trials exploring the use of anti-CD19 CAR T-cells in patients with PCNSL (based on https://clinicaltrials.gov accessed in June 2021)

Clinical phase	Compound	Mechanism of action	Population	ORR	CRR	NCT number	Status
I	CD19CAT-41BBζ CAR T-cells	Anti-CD19 CAR T-cells	R/R	n.a.	n.a.	NCT04443829	Recruiting
I	Tisagenlecleucel	Anti-CD19 CAR T-cells	R/R, 1st line	n.a.	n.a.	NCT04134117	Recruiting
I	Axicabtagene ciloleucel	Anti-CD19 CAR T-cells	R/R	n.a.	n.a.	NCT04608487	Recruiting
I	19(T2)28z1xx CAR T cells	Anti-CD19 CAR T-cells	R/R	n.a.	n.a.	NCT04464200	Recruiting
I	CAR T-cells	Anti-CD19 CAR T-cells	R/R	n.a.	n.a.	NCT04532203	Recruiting
II	Lisocabtagene maraleucel	Anti-CD19 CAR T-cells	R/R	n.a.	n.a.	NCT03484702	Recruiting

ORR, overall response rate; R/R, refractory/relapsed; CRR, complete remission rate; CAR, chimeric antigen-receptor.

ibrutinib with selinexor, a selective inhibitor of nuclear export (SINE) compound that specifically blocks Exportin 1 (XPO1) (195). The combination of the two small molecules has a direct anti-tumor effect but it also able to modulate the tumor microenvironment pushing toward a pro-inflammatory M1-like macrophages and reducing PD1 and SIRPα expression on M2-like macrophages (195).

Using orthotopic patient derived xenografts, Tateishi *et al.* have shown the therapeutic potential of the direct NF-κB inhibition using BAY11-7082 and of the natural compound juglone, an inhibitor of peptidyl-prolyl isomerase PIN1, as anti-lymphoma agents and to sensitize cells to chemotherapy (16). Since NF-κB activation has been associated to low sensitivity to chemotherapy, including high-dose (HD) methotrexate (MTX) (16), inhibition of the pathway, as also achieved with BTK inhibitors, might improve the patient's outcome.

The frequent inactivation of CDKN2A and the high genomic instability suggest the possible benefit of using aurora kinase inhibitors, CDK4/6 inhibitors (196) or PRMT5 inhibitors (197).

As previously mentioned, CNS-DLBCL cells from different patients still recognize the same antigens, and in particular SAMD14 and neurabin-I (11). Bewarder *et al.* applied the B-cell receptor Antigen for Reverse targeting (BAR) approach to develop a molecule that resembles an IgG1 antibody and contains the BCR-binding epitope of the common CNS-DLBCL antigens SAMD14 and neurabin-I instead of variable regions to target the B-cell receptors with specificity for neurabin-I (198). The IgG1-format neurabin-I BAR-body shows *in vitro* ability to induce antibody-dependent cell-mediated cytotoxicity and, based on *in vivo* data obtained with a similar molecule, it might represent a future therapeutic approach (199), although its efficacy and safety still require extensive validations (200).

Due to the expression of BAFF-R on CNS-DLBCL cells and on the role of BAFF signaling in sustaining their growth, antibodies against the soluble BAFF of against BAFF-R have been proposed as potential therapeutic tools for CNS-DLBCL patients (109).

Promoter methylation affecting specific genes can predict the response to specific drugs. Hypermethylation of O6-methylguanine- DNA methyltransferase (MGMT) promoter region has been reported in 30-60% of CNS-DLBCL cases (70,77,84,201,202) and suggests a higher sensitivity to alkylating agents as reported for temozolomide or carmustine (BCNU) in glioma patients (203,204). In five relapsed CNS-DLBCL patients treated with temozolomide, the four responders had methylated MGMT promoter, while the only non-responders had no methylation (202). In a series of 15 CNS-DLBCL cases treated with temozolomide and analyzed for MGMT protein expression, all patients with low expression achieved CR or PR and only one with high MGMT expression responded to treatment (205).

Inversely to MGMT, methylation in the promoter of the gene coding for RFC (reduced folate carrier), involved in MTX cellular uptake, seems to be associated with decreased response to HD-MTX (206). In a series of 37 CNS-DLBCL patients, RFC promoter methylation was detected in 24% of the cases (206). None of patients with promoter methylation were relapse-free at three years and all the patients alive at 3 years had unmethylated RFC (206).

Somatic mutations in the CD19 gene have not yet been reported in CNS-DLBCL patients but the increase in use of anti-CD19 chimeric antigen-receptor (CAR) T-cells in these patients population (*Table 3*), sustained by initial promising reports (207-209), will require its inclusion

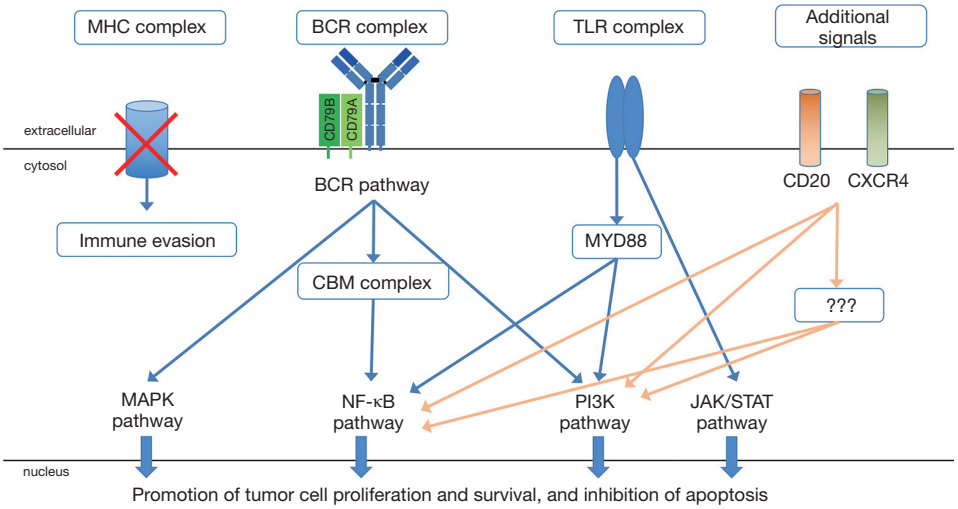

Figure 2 Molecular pathways. Shown are the most important altered pathways for the pathogenesis of CNS-DLBCL. First of all, the tonic active BCR pathway, which in turn activates the MAPK, NF-kB- and PI3K-pathway. Second, as a source of an additional positive signal, is the activation of the TLR pathway, in most cases induced by *MYD88* mutation. *MYD88* is the most often mutated single gene in CNS-DLBCL. Not investigated so often, are all the positive stimulations due to constitutive expressed genes, like *CD20* and *CXCR4*. Probably they could be the basis for new therapeutic strategies. Last, but not least, are pathway, which do not signal any more. Most prominent pathway in the regard the immune evasion via not expressed MHC molecules, due to different mechanism. As such, neo-antigens of the tumor cell cannot be presented to surrounding T cells. CNS-DLBCL, central nervous system diffuse large B-cell lymphoma; CBM, CARD11-BCL10-MALT1; BCR, B cell receptor; TLR, Toll like receptor.

especially in targeted DNA sequencing, especially in follow-up analyses.

It is important to highlight that mutation/methylation screening approach does not identify all the potential therapeutic targets by ignoring surrogate marker. These markers are always expressed by the tumor cells (but not exclusively) and with CD20 as one example are already used as target molecule in therapies. another surrogate marker in CNS-DLBCL is CXCR4, which is always expressed by the tumor cells (210), and never reported mutated in the setting of CNS-DLBCL (211). The ligand CXCL12 is expressed by endothelial cells, microglia cells (only in the infiltration zone) and by the tumor cells itself (210). Perhaps it provides a crucial signal leading to a permissive tumor microenvironment and immune evasion, as described for other cancer types (212), and its targeting might be helpful. Future therapy concepts will indeed focus on personalized targeting. Brain biopsy remains the gold standard for the diagnostic of CNS-DLBCL and it will become the foundation for clinical decisions, although it is important to remind that no single CNS-DLBCL biopsy contains all the hereby described genetic aberrations (*Figure 2*).

In conclusion, our knowledge on the genetic and epigenetic alteration sustaining CNS-DLBCL cells has increased, strengthening the diagnostic criteria and making this disorder a more homogenous disease. Its close link with the systemic MCD/C5 genetic clusters and specific biologic features now provides new opportunities. Novel agents with the capacity to cross the BBB and targeting CNS-DLBCL cells or their tumor microenvironment can be tested and included in future therapeutic schemes. Innovative follow-up approaches can be explored with the use of liquid biopsies and the detection of secreted factors in serum/plasma and/or CSF.

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