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(Article begins on next page)

1 **Genomic profiling of CNS-DLBC suggests novel potential therapeutic targets.**

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53 **ABSTRACT**

54 Primary diffuse large B-cell lymphoma of the CNS (CNS-DLBCL) is an aggressive disease, with
55 dismal prognosis despite the use of high dose methotrexate (MTX)-based polychemotherapy. Our
56 study aimed to expand the biologic profiles of CNS-DLBCL and to correlate them with
57 clinical/imaging findings to gain diagnostic insight and possibly identify new therapeutic targets.
58 We selected 61 CNS-DLBCL whose FFPE samples at first diagnosis were available. These were
59 investigated by immunohistochemistry, cMYC rearrangements were explored by fluorescence in
60 situ hybridization and CNS-DLBCL mutated genes were evaluated by Next Generation Sequencing.
61 CD10, BCL6 and IRF4 were observed in 16%, 83.6% and 93% of cases respectively. As typical of
62 CNS-lymphoma 10/61 (16.4%) cases were classified as germinal center (GCB) and type and 51/61
63 (83.6%) as non-germinal center (non-GCB) type according to the Hans algorithm. Double
64 expression (DE) status for BCL2 and cMYC was detected in 36/61 (59%) cases while 25/61 (41%)
65 were non DE. Rearrangement of the *cMYC* gene was detected in 2 cases, associated with *BCL6*
66 translocation only 1 case. *MYD88*, *PIMI1*, *CD79B* and *TP53* were mutated in 54.5%, 53.5%, 30.2%
67 and 18.4% cases respectively. Novel mutations not previously reported in CNS-DLBCL were
68 found: *AIP* in 23.1%, *PI3KCA* in 15%, *NOTCH1* in 11.4%, *GNAS* 8.1%, *CASP8* in 7.9%, *EGFR* in
69 6.4% *PTEN* in 5.1 and *KRAS* in 2.6%. Survival was significantly longer for patients with mutated
70 *MYD88* (8.7 months vs. 1.7 months; log-rank test = 5.43; p = 0.020) and for patients with mutated
71 *CD79B* (10.8 months vs. 2.5 months; log-rank test = 4.64; p = 0.031). *MYD88* and *CD79B* predicted
72 a longer survival in patients affected by CNS-DLBCL. Notably, we identified novel mutations that
73 enrich mutational landscape of CNS-DLBCL, suggest a role of PTEN-PI3K-AKT and RTK-RAS-
74 MAPK signalling in a subset of CNS-DLBCL and provide new potential therapeutic targets.

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79 **Introduction**

80 Diffuse large B cell lymphoma of the primary central nervous system (CNS-DLBCL) accounts for
81 <3% of brain tumor with a peak incidence between the 5th and 6th decade¹. The most frequent
82 (60%) localization is the supra-tentorial frontal region. Ocular lesions develop concurrently in 20%
83 of patients,^{1,2} while extra-neural dissemination is rare.^{1,3} CNS-DLBCL lesions may be single or
84 multiple, with distinct margins or poorly defined with diffuse parenchyma infiltration .¹ The
85 neoplastic population consists of medium/large proliferating blasts, with a mature B phenotype.¹
86 Most CNS-DLBCL are BCL6⁺/IRF4⁺ and^{1,4} approximately 80% of CNS-DLBCL are
87 BCL2⁺/cMYC⁺ (double expressors), a protein profile that confers poor clinical prognosis; double-
88 hit or triple-hit high grade B-cell lymphomas, with MYC and BCL2 and/or BCL6 rearrangements,
89 are rare.^{5,6} Despite high-dose polychemotherapy and methotrexate (MTX), the prognosis remains
90 poor, with a median overall survival (OS) of about 3 years and a median progression free survival
91 (PFS) of 12 months.⁷ At the bio-pathological level, proliferation and survival of the neoplastic
92 clones are supported by the constitutive activation of the B-cell receptor (BCR), Toll-like receptor
93 (TLR) and NF-kB signaling pathways caused by mutations of *MYD88* and/or *CD79B*.¹ Recently,
94 the landscape of recurrent genetic drivers in DLBCL has been expanded by Schmitz G et al and
95 Chapuy B et al. that uncovered genetic subtypes of DLBCL with distinct clinical characteristics,
96 providing a potential nosology for precision-medicine strategies in DLBCL.^{8,9} These subtypes
97 showed distinct outcomes after immunochemotherapy with the potential to affect the selection of
98 targeted therapies owing to their distinct oncogenic abnormalities. Wright et al revealed a high
99 prevalence of the sub-type termed MCD (based on the co-occurrence of *MYD88* p.L265P and
100 *CD79B* mutations) in primary CNS-DLBCL, defined by gene aberrations involving *MYD88*,
101 *CD79B* and *PIMI*.¹⁰ Their combined genetic, phenotypic, functional, and clinical data suggest that
102 MCD-subtype may be sensitive to BTK, PI3K, BET, BCL2, and JAK inhibitors.¹¹
103 Despite all attempts, the prognosis of CNS-DLBCL remains poor and new therapeutic approaches
104 are needed to improve patient survival. With this background, our aim was to characterize and

105 correlate the biologic profiles CNS-DLBCL to clinical findings and explore new potential
106 therapeutic targets.

107 **Methods**

108 *Patients' selection and tumour samples collection.* From the review of the medical records of the
109 Department of Neurosurgery of IRCCS Istituto Delle Scienze Neurologiche, Bologna, we identified
110 107 pathologically confirmed cases of CNS-DLBCL diagnosed between 2005 and 2020. Of those,
111 sixty-one cases with formalin fixed paraffin embedded (FFPE) adequate material were included in
112 the study. The study was approved by local ethics committee (620/2020/OSS/AUSLBO) and it was
113 performed in accordance with the Declaration of Helsinki. Thirty patients (49.2%) were male and
114 31 females (50.8%), with an age range between 32 and 82 years and a median age of 66 years (IQR:
115 57–72).

116 *Immunohistochemistry.* Paraffin-embedded sections were deparaffinized in HistoClear and
117 dehydrated through graded ethanol. The antigen retrieval was performed in the PT-Link (Agilent
118 Dako, Santa Clara, CA, USA, code PT100/ PT 101), for 5 min at 92°C in EnVision Flex Target
119 retrieval solution High pH (Agilent Dako, code K 8004). Then, tissue samples were incubated at RT
120 for 30 minutes with the following antibodies: CD20 (Agilent Dako 1:300, clone L26, code M0755),
121 CD10 (Leica NewCastle, UK, 1:30, clone 56C6 code CD10-270-L), BCL2 (Abcam, Cambridge ,
122 UK, 1:100, clone E17, code Ab32124), BCL6 (kindly provided by Prof. Falini, indiluted, clone PG-
123 B6p), cMYC (Epitomics, Burlingame, CA 1:80, clone Y69, code 1472-1.), IRF4 (kindly provided
124 by Prof. Falini, 1:3, clone IRF4). Immunostaining has been completed using the Alkaline REAL
125 Detection System Alkaline Phosphatase/RED Rabbit/Mouse (Agilent Dako, code K5005) and
126 chromogen (Fast red), provided with the kit. Finally, slides were counterstained with Hematoxylin,
127 mounted in Glycerine and observed and analysed by Olympus microscope. Slides were observed by
128 2 expert pathologists (CA, SA). Results were recorded as percentage of positive cells and graded as
129 follows: + if >30% positive neoplastic cells, for CD10/BCL6/IRF4 according to Hans' algorithm.

130 [11] A case was defined as cMyc or BCL2 positive if $\geq 40\%$ and $\geq 50\%$ cells were stained
131 respectively.⁶

132 *Genetic analysis.*

133 *Fluorescence in situ hybridization (FISH).* Paraffin embedded tissue were deparaffinized and then
134 pre-treated with two different antigen retrievals. The slides were before incubated in 1mM EDTA
135 buffer (pH 8) in a pressure cooker (9 minutes) and after in Pepsin solution (Sigma Aldrich code
136 P7012) for 14 minutes at 38°C¹². The probes and the samples were denatured at 80 °C for 22 min
137 and then hybridized at 38°C for 22 hours in a hybridizer (Agilent Dako, Santa Clara, CA USA).
138 The experiments were conducted using the probes LSI MYC Dual Color Break Apart
139 Rearrangement (Vysis, Abbott, Downers Grove, Illinois, USA, code 01N63-020, cut-off 3,8%), LSI
140 BCL2 Dual Color Break Apart Rearrangement (Vysis Abbott, code 05N51-020, cut-off 4,7%) and
141 LSI BCL6 dual color Break apart Rearrangement t(3q27) (Vysis Abbott, code 01N23-020, cut off
142 5,5%). Following a stringency washes (0,4XSSC/0,03%NP-40 pH 7,4 solutions at 73°C for 2 min
143 and with 2XSSC/0,1% NP-40 pH 7 solutions at room temperature for 1 min), the slides were
144 mounted and counterstained with DAPI I (Vysis Abbott, code 06J49-001). Microscopical analysis
145 were carried out with an Olympus BX61 microscope and images were recorded using the Cell^F
146 program. *BCL6* and *BCL2* rearrangements were investigated only in cases with translocation of the
147 *cMYC* gene.

148 *Next Generation Sequencing.* DNA from FFPE PCNSL tissue samples was purified by Quick
149 Extract FFPE DNA Extraction Kit (Epicentre, Madison, WI) with some modifications described by
150 Ricci C et al.¹³ DNA mutations were detected using the protocol described previously¹⁴ analysing
151 the following gene panel: *MYD88*, *CD79B*, *PIM1*, *GNAS*, *NOTCH1*, *KRAS*, *PIK3CA*, *EGFR*,
152 *CASP8*, *AIP*, *PTEN*. In brief, after target enrichment by multiplex PCR, libraries with tagged
153 primers were generated using Nextera adapters. Each run on MiSEQ platform (Illumina, Palo Alto,
154 CA) was designed to allocate at least 2K reads/region aimed to have a depth of coverage of at least
155 2000x. FASTQ files were filtered with PHRED quality score > Q30 and length > 100 bp, and reads

156 were mapped in a Galaxy Project environment to the hg38 human reference genome with BWA-
157 MEM, GATK local realignment, HaplotypeCaller and Picard MarkDuplicates.¹⁵ The BAM files
158 were visualized using the Integrative Genomic Viewer (IGV) to identify mutations with Variant
159 Allele Frequency (VAF) of at least 10%; only bidirectional variant calls with more than 10 reads
160 were reported. ¹⁵ Next generation sequencing analysis was available in 46 cases, as fifteen FFPE
161 specimens were over-fixed and not amplifiable. Protein sequence and functional information were
162 obtained by Uniprot database.

163 *Statistical analysis.* Demographic and clinical features were described using absolute frequencies
164 and percentages for categorical variables, mean and standard deviation for quantitative symmetrical
165 variables or median and interquartile range (IQR) for quantitative asymmetrical variables. The
166 associations between immunohistochemistry and radiology, between immunohistochemistry and
167 mutated genes, and between radiology and mutated genes were investigated using the χ^2 test, or
168 Exact Fisher's test when expected cell count is less than 5. The Kaplan–Meier product limit was
169 used to estimate the overall survival (OS) curve. Differences in survival between subgroups were
170 assessed with the log-rank test. Statistical analysis was performed with IBM SPSS version 25.0 and
171 Stata 13. The significance level was set at $p < 0.05$.

172 **Results**

173 *Phenotypic profile.* By immunohistochemistry, diffuse and strong expression of CD20 characterized
174 all 61 (100%) samples. CD10, BCL6 and IRF4 were observed in 10 (16%), 51 (83.6%) and 57
175 (93%) of 61 cases respectively. As typical of CNS-lymphoma 10 of 61 (16.4%) cases were
176 classified as GCB type and 51/61 (83.6%) as non-GCB type according to the Hans algorithm.
177 BCL2 and cMYC proteins were expressed in 51 (83.6%) and 41 (67.2%) of 61 samples
178 respectively. Double expression (DE) status for BCL2 and cMYC was detected in 36 (59%) cases
179 while 25 (41%) were non DE. The immunohistochemical data is summarized in Table 1.

180 *Fish analysis.* FISH analysis was successful in 59 of 61 (96.7%) samples; *cMYC* rearrangements
181 were present in 2 (3.4%) of 59 cases and in one was associated to *BCL6* translocation. This latter
182 harboured *MYD88* mutated (p.L265P).

183 Next generation sequencing showed *MYD88* mutations in 24 (54.5%) of 44, all carrying p.L265P
184 except one case with p.A260T. Both mutations are located in the Toll/IL-1 Receptor (TIR) domain
185 of the protein.

186 Twenty-three cases carried a mutation in proto-oncogene with serine/threonine kinase *PIMI* (23/43,
187 53.5%), with a great variety of different mutations, mostly missense mutations, occurring at protein
188 kinase domain, of which the p.E135K was the most common involving four cases. In 9 samples 2 or
189 3 different *PIMI* mutations were simultaneously present. *CD79B* missense mutations were found in
190 13 cases (of 43, 30.2%), located within immunoreceptor tyrosine-based activation motif (ITAM), 5
191 of which had p.Y196H, 2 cases p.Y196N, 2 p.Y196S, 2 p.Y196C, 1 p.Y196D, and one the rare
192 p.E198G. Concurrent mutations were found: *MYD88*, *PIMI* and *CD79B* in 4 cases, *MYD88* and
193 *PIMI* in 13 cases, *MYD88* and *CD79B* in 9 cases, and *CD79B* and *PIMI* in 8.

194 Seven cases carried missense mutations in *TP53* gene (7 of 38, 18.4%). Additional mutations were
195 found: *NOTCH1* in 4 of 35 (11,4%), *AIP* in 9 of 39 (23,1%), *GNAS* in 3 of 37 (8.1%) and *CASP8*
196 in 3 of 38 (7.9%).

197 *PI3KCA* missense mutations were found in 6 of 40 (15%); in 5 cases they produced an amino acid
198 substitution in the catalytic domain (p.A1046V, p.D1045N, p.G1049S and p.G1049D in two cases).
199 *PTEN* and *EGFR* genes mutations were found in 2 of 39 (5.1%) and 2 of 31 (6.4%) cases
200 respectively, while p.G12D and in CIS p.G13D mutations of *KRAS* gene co-occurred in a single
201 patient (1/39). Table 2 summarizes all these sequencing data.

202 *Clinical findings.* The disease presented with a single lesion in 25 of 61 (41%) of patients and with
203 multiple lesions in 36 of 61 (59%). On MRI, most patients showed deeply located lesions with
204 homogeneous contrast-enhancement (36 of 50, 72%). Multiple enhancing was seen in 53%. Only
205 one of 34 patients with DWI performed showed a high ADC value, all others presented

206 hyperintense diffusion signal. Perfusion T2-w studies was available in 24 patients, 54% with 2 to 7
207 times higher value of rCBV. No significant statistical correlations were observed between
208 biological and clinical parameters and neuroradiological features.

209 Gross total resection was achieved in 13 of 61 (21%), subtotal resection in 21 (35%) and biopsy in
210 27 (44%). Twenty-four patients, whose treatment was known, had undergone the matrix protocol
211 MTX + cytarabine or MATRix regimen. Of the 61 patients included in the study, 57 died during the
212 follow-up, with a median follow-up time of 3.3 months (95% CI: [2.1 - 5.0]). The longest follow-up
213 was 69.2 months. Figure 1 shows the Kaplan-Meier survival curve. One-month, 3-month, 6-month
214 overall survival rates were 88.5% (95% CI: [77.4% - 94.4%]), 52.5% (95% CI: [39.3% - 64.1%]),
215 and 37.7% (95% CI: [25.7% - 49.6%]), respectively.

216 No statistically significant correlation was found between cell of origin GCB/non-GCB, BCL2 and
217 cMYC expression and survival nor between DE or non-DE status and prognosis. Survival was
218 significantly longer for patients with mutated *MYD88* (8.7 months vs. 1.7 months; log-rank test =
219 5.43; p = 0.020) compared to those without *MYD88* mutation, and for patients with mutated *CD79B*
220 (10.8 months vs. 2.5 months; log-rank test = 4.64; p = 0.031) compared to those without *CD79B*
221 mutation.

222 **Discussion**

223 CNS-DLBCL, together with vitreoretinal and testicular DLBCL, is now grouped in a new category
224 called large B-cell lymphoma of the immune-privileged sites.¹⁶ The prognosis of CNS-DLBCL
225 remains poor and new therapeutic approaches are needed to improve patient survival. OS is lower in
226 our series than prior studies, this could be due to patient selection bias, which in our study had a
227 median older age (56 vs 64) and a much higher frequency of multiple lesions (59% vs 30%).¹

228 Similar to previously reported, based on our data, single and double hit rearrangements of MYC
229 gene were rare.^{1,6} Differently from a recent report by Asano et al and Kim et al DE status for BCL2
230 and cMYC expression did not impact on prognosis in our cohort of patients.^{5,17}

231 Genomic studies suggest that cell proliferation and survival in CNS-DLBCL are driven by
232 deregulated TLR and BCR signaling pathways inducing constitutive NFκB activation, with a high
233 frequency of somatic non-synonymous mutations in *MYD88* and *CD79B* genes.¹⁸⁻²⁴ Therefore
234 inhibitors of TLR/BCR signaling such as ibrutinib, blocking Bruton's tyrosine kinase (BTK), was
235 proposed as alternative therapeutic target and seem to be effective in CNS-DLBCL.²⁵⁻²⁸ We found
236 *MYD88* mutations in 54.5% of the cases resulting the most frequently mutated gene in our series;
237 *CD79B* mutations considered one of the hallmark of CNS-DLBCL mutational signature, were
238 demonstrated in 30.2% of the cases. This gene encodes the Ig-beta protein of BCR multimeric
239 complex and its activating mutations reinforce BCR signalling contributing to sensitivity to
240 Ibrutinib. Interestingly, survival was significantly longer for patients with mutated *MYD88* and
241 *CD79B* compared to those with wild type genes. Our findings confirm data reported by Curran et al
242 and Zhou et al respectively, and are in contrast with two studies reporting a poor prognosis
243 associated with the *MYD88* mutation.²⁹⁻³² These conflicting results might reflect a selection bias
244 among small study populations, given the rarity of CNS-DLBCL.

245 *PIM1* was found to be the second most frequently mutated gene (53.5%), with a great variety of
246 different mutations. PIM1 protein belongs to the Ser/Thr protein kinase family which is
247 overexpressed in hematopoietic malignancies and in prostate and breast cancers where it was
248 proposed as new therapeutic target.³³⁻³⁵ Although *PIM1* mutation status was reported to impact the
249 outcome, we did not find any correlation with prognosis.³²

250 *TP53* alterations seem to play a minor role in CNS-DLBCL. Zorofchian et al (26.7%) and other
251 authors have suggested that disruption of the p53-pathway may be associated with poor PFS.^{37,38}
252 In our series *TP53* mutations were not significantly related to prognosis.

253 Notably, we detected for the first time *AIP* mutations in CNS-DLBCL (23,1% of the cases). Aryl
254 hydrocarbon receptor-interacting protein (*AIP*) is a co-chaperone to heat shock proteins and nuclear
255 receptors which behaves as tumor suppressor gene. In colorectal, pancreatic and gastric cancer high
256 expression of *AIP* is associated with tumour development and more aggressive disease and

257 inhibiting aryl hydrocarbon receptor was proposed as potential therapeutic target.³⁹⁻⁴¹ Recently *AIP*
258 was found to be a positive regulator of *BCL6* expression in germinal centers cells, protecting *BCL6*
259 from ubiquitin-mediated proteasomal degradation, and deletion of *AIP* in B cells decrease *BCL6*
260 expression, reducing germinal center B cells and diminishing adaptive immune responses.⁴²
261 Furthermore *AIP* was required for optimal AKT signaling in response to BCR stimulation and
262 seems to be highly expressed in primary DLBCL compared to healthy tissue with implications for
263 the pathobiology of this disease.⁴²

264 The NOTCH signalling pathway is widely involved in cellular proliferation, differentiation, and
265 apoptosis.⁴³ We *NOTCH1* mutations in 11,4% of the samples: *NOTCH1* mutations are distinctive
266 drivers of systemic DLBCL and were not previously described in CNS-DLBCL.⁸

267 *GNAS* and *CASP8* were mutated in 8.1% and 7.9% of the cases, respectively. *GNAS* is the most
268 frequently mutated G-protein in human cancers and activating mutations in the gene *GNAS* have
269 been found in pituitary, thyroid, pancreatic, biliary tract and intestine tumors as well as in Burkitt
270 lymphoma, systemic DLBCL and Hodgkin lymphoma.⁴⁴⁻⁴⁷ Zhou et al reported *GNAI3* mutations to
271 be associated with a shorter PFS and overall survival in primary central nervous system lymphoma
272 patients, however we did not find any correlation with prognosis.⁴⁸ We also identified somatic
273 mutations *CASP8* for which a functional role has not been previously suspected in CNS-DLBCL,
274 although p.R472* nonsense mutation detected in one of our samples was previously reported in
275 systemic DLBCL.⁴⁹ *CASP8* encodes a member of the cysteine-aspartic acid protease (caspase)
276 family and sequential activation of caspases plays a central role in the execution-phase of cell
277 apoptosis. In addition, caspase-8 participates in maintenance of genomic integrity and loss of
278 caspase-8 in B lymphocytes leads to B-cell malignancies; moreover, *CASP8* mutations were found
279 in mantle cell lymphoma.⁵⁰

280 *PI3KCA* missense mutations, previously described in several types of carcinomas,¹ were found in 6
281 of 40 (15%), while *PTEN* mutations were detected in 2 of 39 samples (5.1%). The
282 PI3K/AKT/mTOR is an important pro-survival pathway which plays a pivotal role in the
283 development of malignant tumours being often aberrantly activated in different types of cancer also
284 including lymphomas. Zang et al recently showed that PI3K/AKT/mTOR signaling is aberrantly
285 activated in CNS-DLBCL and correlated with a poor prognosis.⁵¹ They found that p-mTOR
286 expression was an independent risk factor in terms of PFS in patients with CNS-DLBCL.
287 Moreover, Takashima et al found that copy number losses in the PTEN-PI3K-AKT proapoptotic
288 pathway are associated with poor prognosis in CNS-DLBCL patients while PTEN mutation was
289 related to shorter OS in the study of Todorovic et al.^{52,53} PI3K/mTOR inhibitors has also become a
290 potential therapeutic target in CNS-DLBCL. A phase II trial of 37 relapsed/refractory CNS-DLBCL
291 patients demonstrated that temsirolimus had a positive effect with 54% overall response rate, while
292 lower response rate of 25% was seen in a clinical trial targeting using the pan-PI3K inhibitor
293 buparlisib.^{54,55} The use of dual pan-PI3K/mTOR inhibitor bimiralisib (PQR309) in a multicenter
294 phase I/II trial (NCT02669511) as well as BAY80-6946 (copanlisib) in a phase Ib/II clinical trial
295 (NCT03581942) are still under investigation in relapsed/refractory CNS-DLBCL. Moreover,
296 Inhibition of the PI3K isoforms p110 α /p110 δ or mTOR synergized with ibrutinib to induce cell
297 death in CD79B-mutant CNS-DLBCL cells suggesting that combined inhibition of BTK and
298 PI3K/mTOR could overcome the resistance of lymphoma cells to ibrutinib.⁵⁶ So far, no PI3K
299 mutations were described in CNS-DLBCL and our data provide a strong rational for a clinical
300 application of inhibitors targeting the PI3K/ AKT/mTOR signalling pathway at least in a subset of
301 patients.
302 Finally, mutations in the *EGFR* and *KRAS* genes were found in in 6.4% and 2.6% of the cases
303 respectively that could be promising therapeutic targets. Although copy number alterations with

304 amplifications in RTK-RAS-MAPK signalling has been correlated to a poorer prognosis in CNS-
305 DLBCL we didn't find any association with survival.⁵²

306 **Conclusion**

307 *MYD88* and *CD79B* predict a longer survival in patients affected by CNS-
308 DLBCL. The *rearrangements* of the *MYC* gene are rare as well as double hit events involving
309 *BCL6* and/or *BCL2* genes in the present series of CNS-DLBCL. COO and DE status does not affect
310 the prognosis of the 61 CNS-DLBCL cases. Notably, we identified novel mutations that enrich the
311 mutational landscape of CNS-DLBCL, suggests a role of PTEN-PI3K-AKT and RTK-RAS-MAPK
312 signalling in a subset of CNS-DLBCL and that provides new potential therapeutic targets.

313

314 **References**

- 315 1. Kluin PM, et al. Primary diffuse large B-cell lymphoma of the CNS. In: Swerdlow SH, Campo E,
316 Harris NL, et al. (eds). WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues.
317 Lyon, France: IARC Press; 2017; 300-2.
- 318 2. Schlegel. Primary CNS lymphoma. *Ther Adv Neurol Disord* 2:93-104 U (2009).
- 319 3. Deckert M, Engert A, Brück W, Ferreri AJ, Finke J, Illerhaus G, et al. Modern concepts in the
320 biology, diagnosis, differential diagnosis and treatment of primary central nervous system
321 lymphoma. *Leukemia* 25:1797-807 (2011)
- 322 4. Marcus C, Maragkos GA, Alterman RL, Uhlmann E, Pihan G, Varma H. GCB-type is a
323 favorable prognostic factor in primary CNS diffuse large B-cell lymphomas. *J Clin Neurosci* 83:
324 49-55 (2021)
- 325 5. Kim S, Nam SJ, Kwon D, Kim H, Lee E, Kim TM, et al. MYC and BCL2 overexpression is
326 associated with a higher class of Memorial Sloan-Kettering Cancer Center prognostic model and
327 poor clinical outcome in primary diffuse large B cell lymphoma of the central nervous system. *BMC*
328 *Cancer* 16:363 (2016)

- 329 6. Pina-Oviedo S, Bellamy WT, Gokden M. Analysis of primary central nervous system large B-
330 cell lymphoma in the era of high-grade B-cell lymphoma: Detection of two cases with MYC and
331 BCL6 rearrangements in a cohort of 12 cases. *Ann Diagn Pathol* **48**: 151610 (2020)
- 332 7. Abrey LE, DeAngelis LM, Yahalom J. Long-term survival in primary CNS lymphoma. *J Clin*
333 *Oncol* **16**:859–63 (1998)
- 334 8. Schmitz R, Wright GW, Huang DW, Johnson CA, Phelan JD, Wang JQ, et al. Genetics and
335 Pathogenesis of Diffuse Large B-Cell Lymphoma. *N Engl J Med* **378**, 1396–1407 (2018).
- 336 9. Chapuy B, Stewart C, Dunford AJ, Kim J, Kamburov A, Redd RA., et al. Molecular subtypes of
337 diffuse large B cell lymphoma are associated with distinct pathogenic mechanisms and outcomes.
338 *Nat Med* **24**, 679–690 (2018)
- 339 10. Wright GW, Huang DW, Phelan JD, Coulibaly ZA, Roulland S, Young RM, et al. A
340 Probabilistic Classification Tool for Genetic Subtypes of Diffuse Large B Cell Lymphoma with
341 Therapeutic Implications. *Cancer Cell* **37**(4):551-568 (2020)
- 342 11. Hans CP, Weisenburger DD, Greiner TC, Gascoyne RD, Delabie J, Ott G, et al. Confirmation of
343 the molecular classification of diffuse large B-cell lymphoma by immunohistochemistry using a
344 tissue microarray. *Blood* **1**;103(1):275-82 (2004)
- 345 12. Ventura RA, Martin-Subero JI, Jones M, McParland J, Gesk S, Mason DY, et al. FISH analysis
346 for the detection of lymphoma-associated chromosomal abnormalities in routine paraffin-embedded
347 tissue. *J Mol Diagn* **8**(2):141-51 (2006)
- 348 13. Ricci C, Morandi L, Ambrosi F, Righi A, Gibertoni D, Maletta F, et al. Intron 4-5 hTERT DNA
349 Hypermethylation in Merkel Cell Carcinoma: Frequency, Association with Other Clinico-
350 pathological Features and Prognostic Relevance. Multicenter Study. *Endocr Pathol* **32**(3):385-395
351 (2021)
- 352 14. Gabusi A, Gissi DB, Montebugnoli L, Asioli S, Tarsitano A, Marchetti C, et al. Prognostic
353 impact of intra-field heterogeneity in oral squamous cell carcinoma. *Virchows Arch* **476**(4):585-595
354 (2020)

- 355 15. Afgan E, Baker D, van den Beek M, Blankenberg D, Bouvier D, Čech M, et al. The Galaxy
356 platform for accessible, reproducible and collaborative biomedical analyses: 2016 update. *Nucleic*
357 *Acids Res* **8**;44(W1):W3-W10 (2016)
- 358 16. Alaggio R, Amador C, Anagnostopoulos I, Attygalle AD, Araujo IBO, Berti E, et al Leukemia.
359 The 5th edition of the World Health Organization Classification of Haematolymphoid Tumours:
360 Lymphoid Neoplasms. 2022 Jul;36(7):1720-1748.
- 361 17. Asano K, Yamashita Y, Ono T, Natsumeda M, Beppu T, Matsuda K et al . Clinicopathological
362 risk factors for a poor prognosis of primary central nervous system lymphoma in elderly patients in
363 the Tohoku and Niigata area: a multicenter, retrospective, cohort study of the Tohoku Brain Tumor
364 Study Group. *Brain Tumor Pathol* **39**(3):139-150 (2022)
- 365 18. Braggio E, McPhail ER, Macon W, Lopes MB, Schiff D, Law M, et al. Primary central nervous
366 system lymphomas: a validation study of array-based comparative genomic hybridization in
367 formalin-fixed paraffin-embedded tumor specimens. *Clin Cancer Res.***17**:4245–53 (2011)
- 368 19. Montesinos-Rongen, M., Schafer, E., Siebert, R. & Deckert, M. Genes regulating the B cell
369 receptor pathway are recurrently mutated in primary central nervous system lymphoma. *Acta*
370 *Neuropathol.* **124**, 905–906 (2012).
- 371 20. Bruno A, Boisselier B, Labreche K, Marie Y, Polivka M, Jouvét A, et al. Mutational analysis of
372 primary central nervous system lymphoma. *Oncotarget* **5**:5065–75 (2014)
- 373 21. Vater I, Montesinos-Rongen M, Schlesner M, Haake A, Purschke F, Sprute R, et al. The
374 mutational pattern of primary lymphoma of the central nervous system determined by whole-exome
375 sequencing. *Leukemia* **29**:677–85 (2015)
- 376 22. Radke J, Ishaque N, Koll R, Gu Z, Schumann E, Sieverling L, et al. The genomic and
377 transcriptional landscape of primary central nervous system lymphoma. *Nat Commun*
378 **10**;13(1):2558 (2022)

- 379 23. Yamada, S., Ishida, Y., Matsuno, A, Yamazaki, K. Primary diffuse large B-cell lymphomas of
380 central nervous system exhibit remarkably high prevalence of oncogenic MYD88 and CD79B
381 mutations. *Leuk. Lymphoma* **56**, 2141–2145 (2015)
- 382 24. Nayyar N, White MD, Gill CM, Lastrapes M, Bertalan M, Kaplan A, et al. MYD88 L265P
383 mutation and CDKN2A loss are early mutational events in primary central nervous system diffuse
384 large B-cell lymphomas. *Blood Adv* **3**, 375–383 (2019)
- 385 25. Grommes, C., Nayak, L., Tun, H. W, Batchelor, T. T. Introduction of novel agents in the
386 treatment of primary CNS lymphoma. *Neuro-Oncol* **21**, 306–313, (2019)
- 387 26. Soussain C, Choquet S, Blonski M, Leclercq D, Houillier C, Rezai K, et al. Ibrutinib
388 monotherapy for relapse or refractory primary CNS lymphoma and primary vitreoretinal
389 lymphoma: Final analysis of the phase II ‘proof-of-concept’ iLOC study by the Lymphoma study
390 association (LYSA) and the French oculo-cerebral lymphoma (LOC) network. *Eur. J. Cancer* **117**,
391 121–130 (2019)
- 392 27. Lionakis MS, Dunleavy K, Roschewski M, Widemann BC, Butman JA, Schmitz R, et al.
393 Inhibition of B cell receptor signaling by Ibrutinib in primary CNS lymphoma. *Cancer Cell* **31**,
394 833–843 e835 (2017)
- 395 28. Grommes C, Pastore A, Palaskas N, Tang SS, Campos C, Schartz D, et al. Ibrutinib unmasks
396 critical role of Bruton tyrosine kinase in primary CNS lymphoma. *Cancer Discov* **7**, 1018–1029
397 (2017)
- 398 29. Curran OE, Poon MTC, Gilroy L, Torgersen A, Smith C, Al-Qsous W. MYD88 L265P
399 mutation in primary central nervous system lymphoma is associated with better survival: A single-
400 center experience. *Neurooncol Adv* **7**;3(1):vdab090 (2021)
- 401 30. Zhou J, Zuo M, Li L, Li F, Ke P, Zhou Y, Xu Y, et al. PIM1 and CD79B Mutation Status
402 Impacts the Outcome of Primary Diffuse Large B-Cell Lymphoma of the CNS. *Front Oncol*
403 **9**;12:824632 (2022)

- 404 31. Takano S, Hattori K, Ishikawa E, Narita Y, Iwadate Y, Yamaguchi F, et al. MyD88 Mutation in
405 Elderly Predicts Poor Prognosis in Primary Central Nervous System Lymphoma: Multi-Institutional
406 Analysis. *World Neurosurg* **112**:e69-e73 (2018)
- 407 32. Hattori K, Sakata-Yanagimoto M, Okoshi Y, Goshima Y, Yanagimoto S, Nakamoto-Matsubara
408 R, et al. MYD88 (L265P) mutation is associated with an unfavorable outcome of primary central
409 nervous system lymphoma. *Br J Haematol* **177**(3):492-494 (2017)
- 410 33. Mahata S, Sahoo PK, Pal R, Sarkar S, Mistry T, Ghosh S, et al. PIM1/STAT3 axis: a potential
411 co-targeted therapeutic approach in triple-negative breast cancer. *Med Oncol* **15**;39(5):74 (2022)
- 412 34. Zhong S, Peng S, Chen Z, Chen Z, Luo JL. Choosing Kinase Inhibitors for Androgen
413 Deprivation Therapy-Resistant Prostate Cancer. *Pharmaceutics* **24**;14(3):498 (2022)
- 414 35. Zhao Y, Aziz AUR, Zhang H, Zhang Z, Li N, Liu B. A systematic review on active sites and
415 functions of PIM-1 protein. *Hum Cell* **35**(2):427-440 (2022)
- 416 36. Lacy SE, Barrans SL, Beer PA, Painter D, Smith AG, Roman E, et al. Targeted sequencing in
417 DLBCL, molecular subtypes, and outcomes: A Haematological Malignancy Research Network
418 report. *Blood* **135**, 1759–1771 (2020).
- 419 37. Zorofchian S, El-Achi H, Yan Y, Esquenazi Y, Ballester LY. Characterization of genomic
420 alterations in primary central nervous system lymphomas. *J Neurooncol* **140**(3):509-517 (2018)
- 421 38. Munch-Petersen HD, Asmar F, Dimopoulos K, Areškevičiūtė A, Brown P, Girkov MS, et al.
422 TP53 hotspot mutations are predictive of survival in primary central nervous system lymphoma
423 patients treated with combination chemotherapy. *Acta Neuropathol Commun* **22**;4:40 (2016)
- 424 39. Solís-Fernández G, Montero-Calle A, Sánchez-Martínez M, Peláez-García A, Fernández-
425 Aceñero MJ, Pallarés P, et al. Aryl-hydrocarbon receptor-interacting protein regulates tumorigenic
426 and metastatic properties of colorectal cancer cells driving liver metastasis. *Br J Cancer*
427 **126**(11):1604-1615 (2022)

- 428 40. Fernández-Aceñero MJ, Barderas R, Peláez García A, Martínez-Useros J, Díez-Valladares L,
429 Pérez-Aguirre, et al. Aryl hydrocarbon receptor interacting protein (AIP) significantly influences
430 prognosis of pancreatic carcinoma. *Ann Diagn Pathol* **53**:151742 (2021)
- 431 41. Díaz Del Arco C, Estrada Muñoz L, Barderas Manchado R, Peláez García A, Ortega Medina L,
432 Molina Roldán E, et al. Prognostic Role of Aryl Hydrocarbon Receptor Interacting Protein (AIP)
433 Immunohistochemical Expression in Patients with Resected Gastric Carcinomas. *Pathol Oncol Res*
434 **26**(4):2641-2650 (2020)
- 435 42. Sun D, Stopka-Farooqui U, Barry S, Aksoy E, Parsonage G, Vossenkämper A, et al. Aryl
436 Hydrocarbon Receptor Interacting Protein Maintains Germinal Center B Cells through Suppression
437 of BCL6 Degradation. *Cell Rep* **30**;27(5):1461-1471 (2019)
- 438 43. Artavanis-Tsakonas S, Rand MD, Lake RJ. Notch signaling: cell fate control and signal
439 integration in development. *Science* **30**;284(5415):770-6 (1999)
- 440 44. O'Hayre M, Vazquez-Prado J, Kufareva I, Stawiski EW, Handel TM, Seshagiri S, et al. The
441 emerging mutational landscape of G proteins and G-protein-coupled receptors in cancer. *Nature*
442 *reviews Cancer* **13**(6):412–24 (2013)
- 443 45. Love C, Sun Z, Jima D, Li G, Zhang J, Miles R, et al. The genetic landscape of mutations in
444 Burkitt lymphoma. *Nature genetics* **44**(12):1321–5 (2012)
- 445 46. Lohr JG, Stojanov P, Lawrence MS, Auclair D, Chapuy B, Sougnez C, et al. Discovery and
446 prioritization of somatic mutations in diffuse large B-cell lymphoma (DLBCL) by whole-exome
447 sequencing. *Proc Natl Acad Sci U S A* **6**;109(10):3879-84 (2012)
- 448 47. Wienand K, Chapuy B, Stewart C, Dunford AJ, Wu D, Kim J, et al. Genomic analyses of flow-
449 sorted Hodgkin Reed-Sternberg cells reveal complementary mechanisms of immune evasion. *Blood*
450 *Adv* **10**;3(23):4065-4080 (2019)
- 451 48. Zhou Y, Liu W, Xu Z, Zhu H, Xiao D, Su W, et al. Analysis of Genomic Alteration in Primary
452 Central Nervous System Lymphoma and the Expression of Some Related Genes. *Neoplasia*
453 **20**(10):1059-1069 (2018)

454 49. Hakem A, El Ghamrasni S, Maire G, Lemmers B, Karaskova J, Jurisicova A, et al. Caspase-8 is
455 essential for maintaining chromosomal stability and suppressing B-cell lymphomagenesis. *Blood*
456 **12**;119(15):3495-502 (2012)

457 50. Yang P, Zhang W, Wang J, Liu Y, An R, Jing H. Genomic landscape and prognostic analysis of
458 mantle cell lymphoma. *Cancer Gene Ther* **25**(5-6):129-140 (2018)

459 51. Zhang X, Wu Y, Sun X, Cui Q, Bai X, Dong G, et al. The PI3K/AKT/mTOR signaling pathway
460 is aberrantly activated in primary central nervous system lymphoma and correlated with a poor
461 prognosis. *BMC Cancer* **20**;22(1):190 (2022)

462 52. Takashima Y, Sasaki Y, Hayano A, Homma J, Fukai J, Iwadata Y, et al. Target amplicon
463 exome-sequencing identifies promising diagnosis and prognostic markers involved in RTK-RAS
464 and PI3K-AKT signaling as central oncopathways in primary central nervous system lymphoma.
465 *Oncotarget* **8**;9(44):27471-27486 (2018)

466 53. Todorovic BM, Jelcic J, Mihaljevic B, Kostic J, Stanic B, Balint B, et al. Gene mutation profiles
467 in primary diffuse large B cell lymphoma of central nervous system: next generation sequencing
468 analyses. *Int J Mol Sci* **17**(5):683 (2016)

469 54. Korfel A, Schlegel U, Herrlinger U, Dreyling M, Schmidt C, von Baumgarten L, et al. Phase II
470 trial of temsirolimus for relapsed/refractory primary CNS lymphoma. *J Clin Oncol* **34**(15):1757–
471 1763 (2016)

472 55. Grommes C, Pentsova E, Nolan C, Wolfe J, Mellingshoff IK, Deangelis L. Phase II study of
473 single agent buparlisib in recurrent/refractory primary (PCNSL) and secondary CNS lymphoma
474 (SCNSL). *Ann Oncol* **27**(suppl 6):335 (2016)

475 56. Grommes C, Pastore A, Palaskas N, Tang SS, Campos C, Scharz D, et al. Ibrutinib unmasks
476 critical role of bruton tyrosine kinase in primary CNS lymphoma. *Cancer Discov* **7**: 1018–1029
477 (2017)

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