Liquid biopsy for improving diagnosis and monitoring of CNS lymphomas: a RANO review.

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Abstract

Background. The utility of liquid biopsies is well documented in several extracranial and intracranial (brain/leptomeningeal metastases, gliomas) tumors.

Methods. The RANO (Response Assessment in Neuro-Oncology) group has set up a multidisciplinary Task Force to critically review the role of blood and CSF-liquid biopsy in central nervous system lymphomas, with a main focus on primary central nervous system lymphomas (PCNSL).

Results. Several clinical applications are suggested: diagnosis of PCNSL in critical settings (elderly or frail patients, deep locations, steroids responsiveness), definition of minimal residual disease, early indication of tumor response or relapse following treatments and prediction of outcome.

Conclusions. Thus far, no clinically validated circulating biomarkers for managing both primary and secondary CNS lymphomas exist. There is need of standardization of biofluid collection, choice of analytes and type of technique to perform the molecular analysis. The various assays should be evaluated through well organized central testing within clinical trials.

Keywords: CSF biomarkers, MYD88 mutations, circulating tumor DNA, primary CNS lymphomas, secondary CNS lymphomas.

Introduction

Primary CNS lymphoma (PCNSL) in immunocompetent patients has distinctive features as compared to other primary brain tumors of the adult. In the 2022 edition of the WHO Classification of Hematolymphoid Tumors[1] this neoplasm is classified within the group of "Large B cell lymphomas of immuno-privileged sites", whereas it is considered a specific entity in the International Consensus Classification of Mature Lymphoid Neoplasms.[2]

PCNSL is confined to the CNS including brain, spine, cerebrospinal fluid and eyes. It peaks in older ages, is often infiltrative, located in deep brain structures or multicentric, and is typically responsive to chemotherapy with high dose (HD) methotrexate-based regimens and radiotherapy. The standard option for diagnosis is a biopsy,[3] that bears a nonnegligible risk of neurological complications, such as bleeding, due to the anatomic location and/or comorbidities associated with advanced age. Moreover, steroid responsiveness, that occurs in about half of the patients, may render the biopsy unfeasible or at high risk of being inconclusive, thus causing a delay in starting the appropriate treatment. Lastly, the differentiation of PCNSL from glioblastomas or brain metastasis may be difficult based on conventional and advanced neuroimaging. These hurdles explain why a noninvasive procedure, such as liquid biopsy to identify specific biochemical and molecular biomarkers in biofluids, is highly attractive to avoid the need for or to supplement tissue diagnosis. Overall, earlier diagnosis of PCNSL is correlated with a better prognosis,[4] hence there is need of strategies that aid in early diagnosis.

Moreover, as in other solid or hematological malignancies, liquid biopsy may allow a longitudinal monitoring of the outcome following treatments in association with imaging tools.

The RANO group has published reviews on clinical applications of liquid biopsy in CNS metastases[5] and gliomas,[6] respectively, and has set up a multidisciplinary Task Force, including neuro-oncologists, neurosurgeons and hematologists/oncologists to critically review the role of liquid biopsy in CNS lymphomas. The main focus of this manuscript is PCNSL; however, secondary nervous system lymphoma (SNSL) and vitreous lymphoma (VRL), that differ from PCNSL with respect to their biological, molecular and clinical characteristics, will be also briefly discussed because there is in these entities a role for liquid biopsy.

General concepts on neuroimaging

In up to 80% of patients, PCNSLs present on MRI as supratentorial lesions located in the periventricular white matter, corpus callosum, basal ganglia, thalamus and with contact with the ependyma.[7] Lesions commonly exhibit an intense and homogenous contrast-enhancing pattern with vasogenic edema less prominent than in malignant gliomas or brain metastases. Leptomeningeal enhancement is more typical for secondary CNS lymphomas.

Using perfusion-weighted MR imaging (PWI), cerebral blood volume in patients with PCNSL is usually lower than in patients with glioblastoma.[8]

Due to the hypercellularity of PCNSL, water diffusion is often restricted and tumors are hyperintense on diffusion-weighted MR imaging (DWI),[9] while on apparent diffusion coefficient (ADC) maps, lesions are hypointense with low ADC values. Of note, PCNSL may have a more restricted water diffusion and lower ADC values than glioblastomas[10] and brain metastases,[11] and ADC features may be used as a biomarker for response evaluation[12] or prediction of survival.[13]

Similar to glioblastomas and brain metastases, proton MR spectroscopy in patients with PCNSL frequently reveals elevated lipid peaks in absence of necrosis combined with high choline/creatine ratios.[14]

Radiomics-based machine-learning models could further improve the differentiation between PCNSL and glioblastoma.[15,16]

Radiolabeled glucose (2-[^{18}F]-fluoro-2-deoxy-D-glucose; FDG) is the most frequently studied PET radiotracer in patients with PCNSL because of a markedly increased uptake due to an accelerated glycolytic metabolism and high cellular density of lymphoid cells.[17] The clinical value of FDG PET has been evaluated for the characterization of newly diagnosed PCNSL including differential diagnosis (in particular vs malignant gliomas) and outcome prediction before treatment.[17,18] The physiologically increased uptake of FDG in the basal ganglia, thalamus, and grey matter may hamper the detection of an underlying PCNSL. A significantly improved lesion-to-background contrast is provided by radiolabeled amino acids, such as [^{11}C]-methyl-L-methionine (MET), *O*-(2-[^{18}F]fluoroethyl)-L-tyrosine (FET), and 3,4-dihydroxy-6-[^{18}F]-fluoro-L-phenylalanine (FDOPA), that do not require disruption of the blood-brain barrier.[19] MET has been employed for prediction of prognosis and assessment

of treatment response.[20-22] Despite encouraging results in predicting survival time, experience with MET PET is limited, most probably related due to the short half-life of C-11 (20 minutes) and the need of an on-site cyclotron, while amino acids labeled with F-18, such as FET and FDOPA, are increasingly used, but available data remain scarce.[23,24]

Promising investigational PET tracers are ^{68}Ga-Pentixafor and ^{18}F-Fludarabine. In PCNSL and other cancer types, such as leukemia and myeloma, the C-X-C chemokine receptor 4 (CXCR4), a transmembrane chemokine receptor, is overexpressed and therefore a compelling target for PET imaging using the CXCR4-directed tracer ^{68}Ga-Pentixafor. Initial studies suggest a high accuracy for PCNSL detection, with a high contrast between lesions and background activity.[25,26] A more lymphoma-specific tracer seems to be ^{18}F-fludarabine, whose specificity for lymphoma imaging has been demonstrated in an animal study[27] and initial first-in-human studies.[28,29] Importantly, uptake in reactive tissues (e.g., inflammatory processes) seems to be absent.

CSF Biomarkers

Standard CSF diagnostics

The role of CSF studies in PCNSL has been typically reserved for routine diagnostics and response assessment. CSF studies have been utilized for diagnosis 1) in the absence of brain parenchymal lesions or 2) deep lesions not amenable for biopsy or 3) biopsy without confirmation of diagnosis or 4) for patients with comorbidities precluding neurosurgical procedures including stereotactic brain biopsies.

Standard CSF studies include cytology and flow cytometry. Additionally, cell count, protein level and immunoglobulin H gene rearrangement may be evaluated, but these do not necessarily add to diagnostic sensitivity or specificity. CSF cytology can identify lymphoma cells or atypical cells by conventional staining methods. The diagnostic accuracy and sensitivity of cytology ranges from 2%-32% and depends on various factors such as disease burden, adequate quantity and processing of the sample, pre-treatment with corticosteroids, and experience of the cytopathologist. Flow cytometry increases diagnostic sensitivity by detecting monoclonal B cells by immunophenotyping and increases sensitivity of diagnosis by 2-3 times. Table 1 highlights CSF results by cytopathology and flow cytometry in patients with newly diagnosed or recurrent CNS lymphomas (primary and secondary) and those with aggressive lymphomas with high risk of CNS dissemination.[30-37] Retrospective studies have

shown that flow cytometry can detect an abnormal B-cell population with relatively low tumor cell concentration/cellularity (<1% of total cells) compared to cytology (5-20%).[32,33] However, the results from flow cytometry are subject to similar limitations as for cytology, particularly fragility of fewer number of cells found in the CSF when compared to peripheral blood.

IgH gene rearrangement

Clonal B-cell population can be detected using polymerase chain reaction (PCR) for clonally rearranged immunoglobulin genes, particularly in the setting of low CSF volume and cell counts when cytology and flow cytometry are non-diagnostic. Specifically, immunoglobulin heavy chain (IgH) gene clonal rearrangement is used to diagnose and monitor response in PCNSL/B-cell lymphoma. PCR may increase the yield of CSF diagnosis with high specificity of up to 97%.[38] The sensitivity of PCR techniques can vary due to the difficulty in adequate DNA extraction. Corticosteroids may also reduce the yield.[39] A prospective study in 282 patients demonstrated a discordance in PCR and cytology results where 11% versus 16% of patients respectively were found to have CSF involvement.[40] Thus, the search for IgH gene rearrangement can be considered only as a complementary method for diagnostic purposes.

Free light chains

B-cell lymphomas demonstrate clonal restriction and express kappa and lambda immunoglobulin free light chains (FLC). Two studies[41,42] have reported that CSF FLC and ratio of kappa to lambda FLC were significantly higher in patients with CNS lymphomas compared to other non-neoplastic neurologic disorders. In one of these studies with 45 patients with PCNSL (30 other neurologic conditions, 60 normal controls), the optimal cut-off for kappa/lambda ratio was found to be 0.35 and diagnostic sensitivity and specificity were 78% and 72%, respectively.[42] Nonetheless, given CSF FLCs are noted in several conditions other than B-cell CNS lymphomas, including plasma cell disorders like Waldenström's macroglobulinemia and myeloma, amyloidosis, other neurologic disorders such a multiple sclerosis and autoimmune conditions, these can only be used to support the diagnosis of PCNSL in association to other techniques.

Protein biomarkers

Several proteins have been detected in CSF in association with PCNSL, and CSF total protein level is associated with prognosis. Some of these proteins may aid in distinguishing CNS lymphoma from other brain lesions, but validation studies are lacking. These are at best additive in terms of increasing diagnostic sensitivity.

Quantitative proteomic analyses have identified CSF proteins associated with tumor invasion, extracellular matrix, complement inhibitors and other proteins associated with activation of immune response, such as matrix metalloprotease-2, vitronectin, fibulin-3, Factor H, Factor I and clusterin.[43] Additionally, proteins associated with regulation of normal brain function, such as neuronal cell adhesion molecules, cadherin 13, contactin-1 and chromogranin A, were found at lower concentrations than in controls. Antithrombin III (ATIII), that is expressed in CNS lymphoma specimens, was also found in high concentrations in the CSF of CNS lymphoma patients compared to CSF of patients with benign lesions in the brain.[43]

The cytokine interleukin-10 (IL-10), and the chemokine CXCL13, are overexpressed in CNS lymphomas and various retrospective and prospective studies have demonstrated higher concentrations in CSF than in other brain tumors as well brain lesions including inflammatory, infectious and demyelinating disorders (Table 2).[44-52] Regarding IL-6, CSF levels higher than those seen in other brain tumors,[44] or in inflammatory CNS conditions has been reported.[52] Most of the publications agree on the value of IL-10 and few on IL-6, particularly in the context of IL-10/IL-6 ratio, and combination of IL-10 with CXCL13 with varying cut-offs leading to differences in sensitivities and specificities of the tests ranging from 50-95% and 89-100%, respectively. CSF IL-10 and/or CXCL13 levels may drop with response to treatment and subsequently increase at the time of recurrence.[44,45,47,48] High levels of pre- and post-treatment IL-10 may be significantly associated with poor PFS,[44,45,48] or early relapse.[47] In general, no association of with OS has been reported.

The search for IL-10 in the vitreous or aqueous humor is increasingly used in PCNSL with ocular involvement.[53] Other CSF protein biomarkers, such as soluble interleukin-2 receptor (sIL-2R), neopterin, other TH17 cell related cytokines like IL-17A, sTACI, sBCNA, BAFF, APRIL and osteopontin[54-56] may be demonstrated in CNS lymphoma, but are also noted in other CNS conditions: overall, none of these independently allow for making the diagnosis.

Increased levels in the CSF of Beta2-microglobulin (b2m) can be noted in CNS lymphoma and leukemia, or inflammatory, demyelinating or autoimmune conditions, paraneoplastic

neurologic syndromes or infections involving the CNS. By itself, it is not as specific, but can be used with other biomarkers for diagnostic purposes.

While CSF proteins are commonly explored in translational studies as diagnostic or prognostic markers in CNS lymphoma, serum proteins have not been identified as useful biomarkers in this disease. While the level of serum lactate dehydrogenase (LDH) is part of the IELSG prognostic index and frequently used as a parameter for tumor burden in systemic aggressive lymphomas, evidence for its value as a single biomarker in the management of patients with CNS lymphoma is lacking.

Liquid biopsy studies in Primary CNS Lymphomas

MYD88 mutations and circulating tumor DNA

The myeloid differentiation primary response gene 88 (MYD88) encodes an adaptor protein involved in Toll-like receptor (TLR) and IL-1 receptor signaling, that activates the NFKB pathway, which is upregulated in PCNSL.[57] The MYD88 L265P mutation is the most frequent mutation in biopsies of PCNSL, with a frequency ranging from 52% to 88% of cases.[58-63] This mutation is less frequent (30%) in diffuse large B-cell systemic lymphomas[64] and is never found in tissue biopsies from primary brain tumors, in particular glioblastomas, and brain metastases from solid tumors[58]: thus, this mutation is a sensitive and specific biomarker for differentiating PCNSL from other brain tumors. Studies of liquid biopsies at diagnosis of PCNSL looking for MYD88 L265P mutation with PCR-based techniques (Table 3) have shown a sensitivity for the detection in the blood of 4-57%.[65,66] A relatively low frequency of MYD88 mutations in plasma ctDNA compared to that in tumor tissue has been reported.[65] Conversely, the sensitivity for the detection of MYD88 L265P mutation in the CSF is generally higher (63.5%-92%).[63,67-71]

Most studies, comparing PCNSL with either systemic diffuse large B-cell lymphomas or inflammatory diseases or healthy controls, have reported a 100% specificity for the detection of MYD88 L265P mutation in blood or CSF.[60,70,71] However, sporadic cases of positivity for MYD88 L265P mutation in the CSF in patients with multiple sclerosis or other neurological diseases have been reported.[63]

A rapid detection system for the MYD88 L265P mutation using real-time PCR-based on a new technology has been developed for intraoperative diagnosis.[72] This technique was

applied to 10 PCNSL and 14 non PCNSL patients with a specificity of 100%. Interestingly, in 2 patients the MYD88 L265P mutation was detected in the CSF collected intraoperatively in the ventricles with parallel positivity in biopsy samples.

As a general consideration, in order to increase the disease diagnosis, multiple makers may be combined such as MYD88 and CD79B mutations.

A recent study has suggested that combining MYD88 L256P mutation detection and clonality determination on CSF cellular and cell-free DNA improves diagnosis of PCNSL.[73]

MYD88 L256P mutation is also detectable in 69% of vitreous aspirates of vitroretinal lymphomas,[74] and consistency with vitreal cytology exam is high.[75] MYD88 L265P mutation can be detected in aqueous humor of 75% of patients with cytologically proven VRL, with consistent results in acqueous and vitreous samples in 7 of 8 explored eyes.[75] Thus, the detection of MYD88 L265P mutation in the aqueous humor may be considered as an additional diagnostic tool in the diagnosis of PCNSL and PVRL and as a staging procedure to confirm intraocular disease in patients with cerebral lymphoma.

Studies of liquid biopsies at diagnosis of PCNSL looking at ctDNA in plasma with next generation sequencing (NGS) have shown a sensitivity of 24%-78% and a specificity of 96%-100% (Table 4).[76-78] Two studies only[69,78] have analyzed the detection of ctDNA in the CSF with a sensitivity of 63.5% - 100% and a specificity of 97%.

Overall, studies directly comparing performance in matched CSF and blood in the same cohort are still limited.[71,78]

Two factors limit the sensitivity of detection of specific mutations in blood, CSF or vitreous fluid, i.e. the concentration of cfDNA and the methods of analysis. Currently reverse transcription quantitative PCR (RT-qPCR) and NGS are the common techniques used for detection of MYD88 mutations[79] but they have never been compared directly. Also, it is unknown whether the sensitivity is altered by using steroids before blood or CSF collection.

The analysis of correlations between the level of MYD88 mutations and other variables has yielded controversial results. As for CSF the correlation was either positive for tumor volume/diameter[68,70] or negative for tumor size and location (deep versus superficial).[63] Interestingly, Mutter and coworkers (2023)[78] have compared blood versus CSF regarding correlations between clinical variables and ctDNA yield (including MYD88 mutations). They found a positive correlation between levels of ctDNA and tumor volume only for plasma, while CSF levels of ctDNA showed a positive correlation with periventricular location.

Fontanilles et al (2017)[76] found that the presence of the MYD88 L265P mutation in plasma tended to be correlated with a shorter OS, while Watanabe et al (2019)[68] did not find any difference in PFS and OS between MYD88 mutant and MYD88 wild-type patients when analyzing CSF.

In the large series of Mutter et al (2023)[78] patients with ctDNA positivity in plasma before treatment had significantly shorter PFS and OS. Moreover, patients with plasma ctDNA positivity at any timepoint during treatment had significantly inferior PFS and OS.

Overall, the detection of plasma ctDNA could contribute to improve monitoring and enable an individual therapy adjustment.

Two small pilot studies[69,80] have suggested that monitoring of CSF ctDNA (including MYD88 mutations) is useful for the evaluation of the effects of ibrutinib-based combination therapy. A large proportion (10/16) of patients with repeated CSF collection had a complete or near complete (PR> 90%) radiographic response of their measurable disease, and this response was accompanied by a disappearance or dramatic reduction of CSF ctDNA. In the remaining patients a rapid disease progression occurred after an initial tumor response and a persistence of ctDNA mutations was observed.

Micro-RNAs

Micro-RNAs (miRNAs) are non-coding RNA regulating gene expression that may be found in CSF from patients with CNS lymphomas, but may be noted in CSF from gliomas, brain metastases, inflammatory and demyelinating brain lesions. In a study of 23 patients with PCNSL and 30 controls with different neurological disorders, miR-21, miR-19 and miR-92a expression levels were higher in PCNSL[81]: the diagnostic tree of the three CSF biomarkers had a sensitivity of 95.7% and a specificity of 96.7%. These findings were confirmed in a larger cohort of 39 patients with PCNSL where miRNA levels correlated with tumor volume and disease status.[82] In another study, similar miRNA levels were found in the CSF but with lower concentrations.[83] Additionally, expression of miR-9, miR-155, miR-125b and miR-196b was noted in CNS lymphoma tissue samples, however, these did not correlate with elevated CSF levels and did not help distinguish CNS lymphoma from other brain lesions.

The yield of micro-RNA from blood is controversial. Baraniskin et al, 2011[81] did not find significant differences in serum micro-RNA between PCNSL (14) and control patients (8).

Conversely, Mao et al (2014)[84] reported that the level of miR-21 in serum was significantly higher in PCNSL than in control patients.

An increasing number of other micro-RNAs was identified and suggested as potential biomarkers for diagnosis and prognosis[85,86]; however, their value should be further validated in prospective studies.

Methylation biomarkers

Epigenetic gene modulation by CpG site methylation has been implicated as one of the important mechanisms in the tumorigenesis: as a result, DNA methylome profiling has been widely utilized in biomarker discovery studies for tumor classification and diagnosis in a myriad of cancers, including PCNSL.[87-89] Initial analysis of 25 PCNSL by Chu et al (2006)[90] showed that promoter methylation of DAPK and MGMT are associated with downregulated protein expression. Methylation of reduced folate carrier (RFC) gene was shown to portend lower post-chemotherapy complete remission rates.[91] Hypermethylation or homozygous deletion of $p14^{ARF}$ and $p16^{INK4a}$ (CDKN2A) has been demonstrated to be a key mechanism in PCNSL tumorigenesis.[87,92,93] DNA methylation profiling has shown no major differences between PCNSL and systemic DLBCL.[88,94]

Despite the wealth of literature on the biological roles of DNA methylation in PCNSL, reports investigating the potential clinical adoption of methylated cell-free DNA (cfDNA) as a liquid biomarker in PCNSL are still limited. In an effort to develop a liquid biomarker assay for PCNSL, Downs et al (2021)[94] investigated DNA methylome data from 48 DLBCL and 656 glioblastoma and lower-grade gliomas by first utilizing The Cancer Genome Atlas (TCGA) database. The authors were able to identify 8 methylation markers that could reliably distinguish PCNSL from eight other malignant primary CNS tumors with high area under the ROC curve (AUC) value of 1.00. Not only were these methylation markers specific to B-cell lymphomas as compared to normal B cells, but they were also expressed at higher levels in PCNSL than normal brain tissue or other blood components. These 8 CpG sites were later verified in the Gene Expression Omnibus (GEO) of 95 PCNSL and 2112 other CNS tumors comprising of 11 different subtypes, and this again demonstrated high discriminative value (AUC, 0.989 to 1.00, $P<0.001$). In addition, the authors developed a Tailed Amplicon Multiplexed-Methylation-Specific PCR (TAM-MSP) assay using two-marker panels comprising of SCG3 and cg054 that showed 100% accuracy and were amplified most efficiently. In a sample cohort of archival formalin fixed, paraffin embedded (FFPE) tissues of 25 PCNSL and 25 other CNS tumors (8 different tumor types) from Wuhan, they found that

the cumulative methylation index (CMI) detected in PCNSL is significantly higher than the other CNS tumors using this assay (AUC 1.00, CI 0.95-1.00, P<0.001). Notably, this methylated biomarker was detected in 20% (3 out of 15) of newly diagnosed PCNSL patients' plasma. This result demonstrates the potential of using plasma DNA methylation signature as a minimally invasive approach to diagnose PCNSL at a timely fashion.

Although global DNA methylation profiling is a valuable tool for cancer biology discovery, the transition of its utilization into clinical use as liquid biomarker has been encumbered by multiple issues. First, plasma is made up of a mixture of components, with each cellular component having its distinct methylation mark as compared to a single lineage cell line which often has only one marker.[95] While methylation-based deconvolution is powerful at identifying tissue-of-origin of the cfDNA and estimating disease burden, the change in composition of different DNA methylation markers over time can easily confound the analysis. Furthermore, the tumor is composed of a heterogeneous population of cancer cells, meaning different methylation levels at the same locus, and setting a threshold of detection in this analysis is often subjective and challenging.[95]

The specificity of epigenetic alterations can be diminished due to changes observed in healthy individuals that occur as a random or a physiologic event due to aging or environmental exposure.[96,97] To optimize detection of markers on cfDNA sample, the primer design also requires special consideration of the DNA fragment size that is present in plasma which is frequently smaller (approximately 165bp) than cellular DNA.[98]

Whole genomic bisulfite sequencing (WGBS) is a well-established method to profile genome-wide DNA methylome[99], but it is more demanding in biofluid samples, due to both the heterogeneity of the cfDNA and the low amount of DNA. To overcome these limitations, several next-generation sequencing (NGS)-based methods have been developed.[100-103] Future prospective studies will be required to understand which of these promising approaches will provide the greatest clinical utility in diagnosing and monitoring PCNSLs.

CSF methylome-based liquid biopsy could reliable classify the 3 major malignant brain tumors (CNSL, glioblastoma and brain metastasis)[104]; moreover, CSF-based features seem able to cluster tumor types from non-neoplastic controls.

Liquid biopsy studies in Secondary Lymphomas of the brain

General concepts on CNS involvement in systemic lymphomas

Central nervous system (CNS) involvement in systemic lymphoid malignancies, i.e. secondary CNS lymphoma (SCNSL), is a rare event and includes three distinct clinical scenarios: (i) synchronous CNS and systemic lymphoma in treatment-naïve patients (TN-SCNSL), (ii) isolated CNS relapse following treatment for systemic lymphoma (relapsed isolated CNSL; RI-SCNSL), and (iii) synchronous CNS and systemic relapse following treatment for systemic lymphoma (RC-SCNSL).[105] The frequency of synchronous SCNSL at presentation varies substantially between different entities. While up to 5% of patients with aggressive Non-Hodgkin lymphomas (NHL) present as TN-SCNSL, CNS dissemination of systemic disease is exceptionally uncommon in patients with indolent NHL.[105-107] Overt secondary CNS involvement of lymphoid malignancies is generally associated with poor prognosis and treatment is heterogenous with no standard of care.[107,108] Conventional analysis of CSF by cytopathology is characterized by low detection rates of lymphoma cells, whereas the concomitant use of flow cytometry results in a 10-fold improvement of sensitivity, in particular in samples with small numbers of nucleated cells.[109-111] Molecular methods for minimal-invasive identification of SCNSL from CSF or blood plasma/serum include various analytes, including proteins, microRNAs, and circulating tumor DNA (ctDNA). The majority of studies investigating the use of liquid biopsy technologies in patients with overt CNS involvement focus on PCNSL, whereas only a few studies have explored biomarkers exclusively in SCNSL, and usually in small case series.

Based on the observation that certain protein concentrations are elevated in the CSF of patients with CNS lymphoma (e.g., CXCL13, IL6, IL10 soluble IL2 receptor, soluble CD27, lambda/kappa free light chains, or beta-2-microglobulin), early studies examined their role as diagnostic biomarkers, discriminating CNSL from other primary or metastatic brain tumors and inflammatory/infectious conditions. In one of the largest studies involving 220 patients (of whom 23 with overt TN- and RC-SCNSL), Rubenstein et al[45] demonstrated that elevated CSF levels of CXCL13 and IL10 as single markers or the combination of both above a certain threshold significantly increased the sensitivity for the detection of SCNSL compared to conventional CSF tests (61-74%), with high specificities between 95% and 99%. In general, the performance of methods investigating protein biomarkers in CSF is highly variable and a robust interpretation of their value for clinical use is challenging due to low sample sizes, various thresholds and lack of independent validation.[78,112]

In more recent studies, research groups focused on circulating nucleic acids such as microRNAs and circulating tumor DNA (ctDNA) to identify overt SCNSL. Krsmanovic et al (2022)[113] leveraged combinations of five microRNAs (miR-19a, miR-20a, miR-21, miR-92a and miR-155) to establish various indices ('oncomiR') for CNSL detection from CSF and plasma in different clinical situations and lymphoma entities. While these indices showed high sensitivities and specificities in most lymphoma types, the plethora of microRNA combinations to create these indices and lack of independent validation introduce the risk of overfitting and overestimation of the performance. In addition, some of these microRNAs are also expressed in other brain tumors (e.g. gliomas) and cancers that frequently metastasize to the brain (breast and lung cancer), limiting their use to diagnose CNS lymphoma.[45,114-116] In contrast, ctDNA molecules carry tumor-specific information that distinguishes CNS lymphomas from other cancer types or brain diseases, including immunoglobulin (Ig) clonotypes or hotspot mutations in genes such as *MYD88* or *CD79B*. Several research groups have explored PCR- or NGS-based technologies to sensitively detect lymphoma-specific ctDNA in CSF or plasma of SCNSL patients.[78,117-119] While the performance of these assays is generally better compared to other analytes, patient cohorts are small and there is a risk that sequencing errors or biological genetic background, such as clonal hematopoiesis of indeterminate potential (CHIP) confine specificity.[120,121]

Table 5 summarizes the studies on biomarkers for SCNSL.[122-125]

Detection of occult CNS involvement and prediction of CNS relapse

The prognosis of patients with lymphoma relapse in the CNS compartment is poor, with a median survival of only 2-5 months.[126-127] Most of relapses occur during or shortly after first-line therapy, suggesting asymptomatic occult CNS involvement at lymphoma diagnosis that remains undetected by conventional diagnostic tools including magnetic resonance imaging (MRI) and CSF cytology or flow cytometry.[119,128] Thus, algorithms, such as the CNS-IPI that relies on six clinical measures to estimate the risk of future CNS relapse at diagnosis of systemic lymphomas, are widely used to inform treatment decisions.[127] This classifier stratifies patients into three distinct risk groups, the high-risk group with a ~10.2% 2-year risk of CNS relapse, the intermediate (3.4%), and low risk groups (0.6%)[127]. Several other factors, such as the involvement of testes or breasts, BCL2/MYC dual expression, the activated B-cell-like (ABC) / Non-germinal center B-cell-like (Non-GCB) cell-of-origin, or the MCD DLBCL subtype are also associated with a higher risk of CNS relapse.[129-133] Prophylactic high-dose intravenous methotrexate is often used for patients with DLBCL at high risk of CNS relapse, yet there is currently limited evidence on its efficacy for the

prevention of CNS events.[134-136] In addition, treating all patients with unfavorable CNS-IPI scores with intensified regimens would result in overtreatment and a significant increase of severe treatment-related toxicities in ~ 90% of high-risk patients who do not experience CNS relapse.[135]

Liquid biopsy investigating CSF- and blood-derived analytes might help to better identify those patients with occult CNS involvement and enable risk-adapted therapeutic strategies. Krsmanovic et al (2022)[113] in an exploratory analysis utilized concentrations of two microRNAs (miR-19a and miR-92a) in plasma at diagnosis of systemic DLBCL to predict CNS relapse in 72 patients. While the predictive capacity of these microRNA levels alone was similar to the CNS-IPI, the combination of both markers identified patients with a particularly high risk for future CNS relapse (51.5% after 4 years in patients with high microRNA levels and high CNS-IPI). In a study that explored the presence of clonotypic ctDNA in the CSF of 22 treatment-naïve systemic B-cell lymphomas with high CNS-IPI by immunoglobulin high-throughput sequencing (IgHTS), 36% of patients tested positive by the assay.[119] Detection of CSF-ctDNA in these patients was associated with a 29% cumulative risk of CNS recurrence after one year, compared to 0% for ctDNA-negative patients. These results suggest a future role of liquid biopsy-guided risk stratification and selection of patients for CNS prophylaxis; yet an independent validation in large prospective patient cohorts is needed to confirm the role of circulating analytes in blood or CSF for the detection of occult CNS involvement.

In conclusion, liquid biopsy technologies represent promising approaches for minimal-invasive detection of overt and occult SCNSL and may provide helpful strategies for personalized treatment selection in the future. Currently, our experience in this field relies on retrospective and unvalidated research studies and prospective testing in larger patient cohorts is required.

New biochemical or molecular alterations for liquid biopsy in PCNSL

Whole genome sequencing and transcriptional profiling has led to the identification of many molecular aberrations that distinguish PCNSL from systemic diffuse large B-cell lymphoma (DLBCL),[137-140] and studies are ongoing to identify these in the CSF from patients with PCNSL. In a recent study the integration of genome-wide data with multi-omic data has revealed the existence of 4 molecular subgroups in PCNSL with a distinct prognostic impact,

that could provide basis for future clinical stratification and subtype-based targeted intervention.[141]

PCNSL genomes are enriched for protein coding mutations that involve mediators of NF-kB activation, the archetypical driver of the ABC subtype of DLBCL. In addition to MYD88 mutations, mutations involving the immunoreceptor tyrosine-based activation motif (ITAM) of CD79B, a protein associated with the B-cell antigen receptor, are present in approximately 40% of PCNSL. Mutational frequencies of CD79B are higher in PCNSL compared to non-CNS, ABC subtype DLBCL. Of note, CD79B mutations are not just restricted to the more frequent ABC subtype of PCNSL, but also noted in GCB subtype. Other aberrations involving the NF-kB pathway in PCNSL include mutations involving CARD11 (Caspase Recruitment Domain Family Member 11) that are detected in ~16% of PCNSL. Somatic mutations involving TNFAIP3 (Tumor Necrosis Factor alpha-induced protein 3), also known as A20, a negative regulator of NF-kB, are detected in up to 15% of PCNSL. Transcriptional profiling analyses of PCNSL have identified upregulated expression of PIM kinases, JAK1, IRF-4 and XBP-1, supporting a key role for NF-kB.[142] Upregulated expression of MYC and increased MYC protein have also been identified. Upregulation of key miRNAs linked to the MYC pathway (miR-17-5p, miR-20a, miR-9) are established.[143] PIM1, a cooperating factor with MYC in lymphomagenesis, is a target of aberrant somatic hypermutation in PCNSL. Another common genomic aberration in PCNSL involves broad deletions on chromosome 6q. Notably, 6q21-23 deletions occur in ~ 40%-60% of PCNSL. Tumor suppressors on 6q include A20 (TNFAIP3) and PRDM1, regulator of B-cell differentiation. Gain in DNA copy number for MALT1, a protease that inactivates A20, also promotes NF-kB pathway signaling. PCNSLs commonly exhibit copy number losses at 9p21.3 involving the cell cycle regulator CDKN2A (~70-80% of cases). Importantly, genomic copy number losses at 6p21, harboring loci for HLA, are frequent in ~ 50% of cases. The significance of TET2 mutations, mainly associated with T-cell lymphomas are detected in about 10% of DLBCL including PCNSL.[144]

Other CSF biomarkers have been recently suggested to hold importance in PCNSL. Six metabolites have been reported to predict the outcome after HD-MTX-based chemotherapy.[145] Elevated CSF lactic acid and increased kynureine/tryptophan ratio correlate with resistance to lenalidomide.[146]

The level of CSF soluble PDL1 (sPDL1) has been associated with poor survival[147]; moreover, patients with PCNSL with high levels of sPDL1 had a higher risk of deep brain and leptomeningeal involvement. These findings could suggest a potential role of sPDL1 as a predictive biomarker for response to immune checkpoint blockade.

Evaluation of specific key mutations, chromosomal rearrangements and DNA copy number alterations associated with PCNSL in the CSF after comparing with genetic profile of tumor biopsy, may better serve to understand the frequency of mutations shared between tumor and CSF ctDNA, and aid diagnosis or assessment of minimal residual disease upon treatment as well as prognostic factors.

Potential clinical applications of Liquid Biopsy in PCNSL

This review suggests several potential applications of liquid biopsies in clinical trials or daily practice of PCNSL patients (Table 6, 7). Liquid biopsies may help establish a diagnosis when tissue biopsy cannot be safely performed in high risk situations, such as lesions located in deep brain structures or elderly or frail patients with comorbidities. Also in patients, who are candidate for resection, due to a favorable location of tumor, liquid biopsies could be of help in the decision-making for the extent of tumor removal when a differential diagnosis between PCNSL and malignant glioma is required, as in the former a biopsy is the standard procedure while in the latter an extensive resection is of prognostic importance.

This could drive also the use of different consolidation options according to the ctDNA response after induction chemo – immunotherapy, with the saving of unnecessary toxicity to many patients. Moreover, when a significant response to steroids with shrinkage of tumor has occurred, liquid biopsy could spare an unrevealing biopsy and, if positive, allow proceeding to the oncological treatment of choice. In this regard, investigations on the usefulness of rapid detection panels for a preoperative diagnosis of PCNSL must be pursued.

Liquid biopsies will be of significant importance for the longitudinal monitoring of a potentially chemotherapy- and radiotherapy-responsive disease such as PCNSL, either by defining a molecular response to treatments in addition to radiological response on MRI, or allowing a close surveillance of the minimal residual disease. Moreover, in the future, the identification of patients at low and high risk for resistance or disease progression after first line treatments could provide opportunities for adjustments of the current therapy.

The sensitivity of CSF ctDNA-based mutational analysis in PCNSL seems superior than that of plasma. This could be in part related to the interference of the BBB with the shedding of tumor-derived DNA into the bloodstream with the risk of concentrations too small for detection. Moreover, some confounding factors may occur in interpreting the results of

plasma ctDNA analysis in patients with PCNSL. cfDNA may carry non tumor-derived somatic mutations, and both NGS and ddPCR may detect low levels of MYD88/CD79B mutations in healthy individuals that tend to increase with age.[66,130] The sensitivity and specificity of an assay is critical for cfDNA measurement to minimize false positive results especially in cancers with lower prevalence such as PCNSL, as it is often diluted in biofluid compartment. Thus, there is need for observational studies that include an adequate number of normal samples from healthy controls to calibrate liquid biopsy approaches. Yet, technical and bioinformatic advances, that significantly reduce background noise, have led to increased sensitivity and specificity of ctDNA detection in solid and hematological cancers, including CNS lymphoma.[78,120,148-151]

Regarding clinical trials the implementation of liquid biopsy by using CSF as biofluid should be easier in CNS lymphomas than in other primary brain tumors of the adult since CSF examinations are part of the standard staging procedures of a lymphomatous disease involving the CNS.[152] All trials should collect and bank blood and CSF liquid biopsies. If there will be better assays in the future the samples will already be available. A better knowledge of correlations between liquid biopsy findings and neuroimaging aspects at different timepoints of the disease course should help establish new integrated criteria for diagnosis and monitoring.

Thus far, no clinically validated circulating biomarkers for managing PCNSL and SCNSL patients exist, due mainly to the small sample size and heterogeneity of patients' cohorts and techniques across the different studies. There is need for standardization of biofluid collection and type of technique to perform the molecular analysis: in particular, the various assays should be evaluated through well-organized centralized testing. Furthermore, enhancing the level of biomarkers in blood and CSF via blood-brain barrier disruption with MRI-guided focused ultrasound is worth investigating.[153]

All the aforementioned issues should be addressed in clinical trials (Table 8) to enable the entry of liquid biopsy approaches into clinical practice.

References:

1. Alaggio R, Amador C, Anagnostopoulos I, et al. The 5th edition of the World Health Organization Classification of Haematolymphoid Tumours: Lymphoid Neoplasms. Leukemia. 2022;36(7):1720-1748.
2. Campo E, Jaffe ES, Cook JR, et al. The International Consensus Classification of Mature Lymphoid Neoplasms: a report from the Clinical Advisory Committee. Blood. 2022;140(11):1229-1253.
3. Abrey LE, Batchelor TT, Ferreri AJ, et al. Report of an international workshop to standardize baseline evaluation and response criteria for primary CNS lymphoma. J Clin Oncol. 2005;23(22):5034-5043.
4. Steffanoni S, Calimeri T. Narrative review: biomarkers, a hope towards early diagnosis in primary CNS lymphoma. Ann Lymphoma. 2021;5:1-14.
5. Boire A, Brandsma D, Brastianos PK, et al. Liquid biopsy in central nervous system metastases: a RANO review and proposals for clinical applications. Neuro Oncol. 2019;21(5):571-584.
6. Soffietti R, Bettegowda C, Mellinghoff IK, et al. Liquid biopsy in gliomas: A RANO review and proposals for clinical applications. Neuro Oncol. 2022;24(6):855-871.
7. Haldorsen IS, Espeland A, Larsson EM. Central nervous system lymphoma: characteristic findings on traditional and advanced imaging. AJNR Am J Neuroradiol. 2011; 32(6):984-992.
8. Hartmann M, Heiland S, Harting I, et al. Distinguishing of primary cerebral lymphoma from high-grade glioma with perfusion-weighted magnetic resonance imaging. Neurosci Lett. 2003; 338(2):119-122.
9. Calli C, Kitis O, Yunten N, Yurtseven T, Islekel S, Akalin T. Perfusion and diffusion MR imaging in enhancing malignant cerebral tumors. Eur J Radiol. 2006; 58(3):394-403.
10. Toh CH, Castillo M, Wong AM, et al. Primary cerebral lymphoma and glioblastoma multiforme: differences in diffusion characteristics evaluated with diffusion tensor imaging. AJNR Am J Neuroradiol. 2008; 29(3):471-475.
11. Stadnik TW, Chaskis C, Michotte A, et al. Diffusion-weighted MR imaging of intracerebral masses: comparison with conventional MR imaging and histologic findings. AJNR Am J Neuroradiol. 2001;22(5):969-976.

12. Huang WY, Wen JB, Wu G, Yin B, Li JJ, Geng DY. Diffusion-Weighted Imaging for Predicting and Monitoring Primary Central Nervous System Lymphoma Treatment Response. AJNR Am J Neuroradiol. 2016;37(11):2010-2018.
13. Hatzoglou V, Oh JH, Buck O, et al. Pretreatment dynamic contrast-enhanced MRI biomarkers correlate with progression-free survival in primary central nervous system lymphoma. J Neurooncol. 2018;140(2):351-358.
14. Harting I, Hartmann M, Jost G, et al. Differentiating primary central nervous system lymphoma from glioma in humans using localised proton magnetic resonance spectroscopy. Neurosci Lett. 2003; 342(3):163-166.
15. Bathla G, Priya S, Liu Y, et al. Radiomics-based differentiation between glioblastoma and primary central nervous system lymphoma: a comparison of diagnostic performance across different MRI sequences and machine learning techniques. Eur Radiol. 2021;31(11):8703-8713.
16. Guha A, Goda JS, Dasgupta A, et al. Classifying primary central nervous system lymphoma from glioblastoma using deep learning and radiomics based machine learning approach - a systematic review and meta-analysis. Front Oncol. 2022;12:884173.
17. Rozenblum L, Houillier C, Soussain C, et al. Role of Positron Emission Tomography in Primary Central Nervous System Lymphoma. Cancers (Basel). 2022;14(17):4071.
18. Zhang G, Li J, Hui X. Use of 18F-FDG-PET/CT in differential diagnosis of primary central nervous system lymphoma and high-grade gliomas: A meta-analysis. Front Neurol. 2022; 13:935459.
19. Langen KJ, Galldiks N, Hattingen E, Shah NJ. Advances in neuro-oncology imaging. Nat Rev Neurol. 2017; 13(5):279-289.
20. Jang SJ, Lee KH, Lee JY, et al. (11)C-methionine PET/CT and MRI of primary central nervous system diffuse large B-cell lymphoma before and after high-dose methotrexate. Clin Nucl Med. 2012; 37(10):241-244.
21. Ahn SY, Kwon SY, Jung SH, et al. Prognostic Significance of Interim 11C-Methionine PET/CT in Primary Central Nervous System Lymphoma. Clin Nucl Med. 2018; 43(8):259-264.
22. Miyakita Y, Ohno M, Takahashi M, Kurihara H, Katai H, Narita Y. Usefulness of carbon-11-labeled methionine positron-emission tomography for assessing the

treatment response of primary central nervous system lymphoma. Jpn J Clin Oncol. 2020; 50(5):512-518.

23. Puranik AD, Boon M, Purandare N, et al. Utility of FET-PET in detecting high-grade gliomas presenting with equivocal MR imaging features. World J Nucl Med. 2019; 18(3):266-272.
24. Salgues B, Kaphan E, Molines E, Brun G, Guedj E. High 18F-FDOPA PET Uptake in Primary Central Nervous System Lymphoma. Clin Nucl Med. 2021; 46(1):59-60.
25. Herhaus P, Lipkova J, Lammer F, et al. CXCR4-Targeted PET Imaging of Central Nervous System B-Cell Lymphoma. J Nucl Med. 2020; 61(12):1765-1771.
26. Starzer AM, Berghoff AS, Traub-Weidinger T, et al. Assessment of Central Nervous System Lymphoma Based on CXCR4 Expression In Vivo Using 68Ga-Pentixafor PET/MRI. Clin Nucl Med. 2021; 46(1):16-20.
27. Hovhannisyan N, Fillesoye F, Guillouet S, et al. [(18)F]Fludarabine-PET as a promising tool for differentiating CNS lymphoma and glioblastoma: Comparative analysis with [(18)F]FDG in human xenograft models. Theranostics. 2018; 8(16):4563-4573.
28. Chantepie S, Hovhannisyan N, Guillouet S, et al. (18)F-Fludarabine PET for Lymphoma Imaging: First-in-Humans Study on DLBCL and CLL Patients. J Nucl Med. 2018; 59(9):1380-1385.
29. Postnov A, Toutain J, Pronin I, et al. First-in-Man Noninvasive Initial Diagnostic Approach of Primary CNS Lymphoma Versus Glioblastoma Using PET With 18 F-Fludarabine and l -[methyl- 11 C]Methionine. Clin Nucl Med. 2022; 47(8):699-706.
30. Finn WG, Peterson LC, James C, Goolsby CL. Enhanced detection of malignant lymphoma in cerebrospinal fluid by multiparameter flow cytometry. Am J Clin Pathol. 1998;110(3):341-346.
31. French CA, Dorfman DM, Shaheen G, Cibas ES. Diagnosing lymphoproliferative disorders involving the cerebrospinal fluid: increased sensitivity using flow cytometric analysis. Diagn Cytopathol. 2000;23(6):369-374.
32. Hegde U, Filie A, Little RF, et al. High incidence of occult leptomeningeal disease detected by flow cytometry in newly diagnosed aggressive B-cell

lymphomas at risk for central nervous system involvement: the role of flow cytometry versus cytology. Blood. 2005;105(2):496-502.

33. Quijano S, Lopez A, Manuel Sancho J, et al. Identification of leptomeningeal disease in aggressive B-cell non-Hodgkin's lymphoma: improved sensitivity of flow cytometry. J Clin Oncol. 2009;27(9):1462-1469.
34. Kiewe P, Fischer L, Martus P, Thiel E, Korfel A. Meningeal dissemination in primary CNS lymphoma: diagnosis, treatment, and survival in a large monocenter cohort. Neuro Oncol. 2010;12(4):409-417.
35. Schroers R, Baraniskin A, Heute C, et al. Diagnosis of leptomeningeal disease in diffuse large B-cell lymphomas of the central nervous system by flow cytometry and cytopathology. Eur J Haematol. 2010;85(6):520-528.
36. Benevolo G, Stacchini A, Spina M, et al. Final results of a multicenter trial addressing role of CSF flow cytometric analysis in NHL patients at high risk for CNS dissemination. Blood. 2012;120(16):3222-3228.
37. Stacchini A, Aliberti S, Demurtas A, Benevolo G, Godio L. Ten antibodies, six colors, twelve parameters: a multiparameter flow cytometric approach to evaluate leptomeningeal disease in B-cell non-Hodgkin's lymphomas. Cytometry B Clin Cytom. 2012;82(3):139-144.
38. Ekstein D, Ben-Yehuda D, Slyusarevsky E, Lossos A, Linetsky E, Siegal T. CSF analysis of IgH gene rearrangement in CNS lymphoma: relationship to the disease course. J Neurol Sci. 2006;247(1):39-46.
39. Gleissner B, Siehl J, Korfel A, Reinhardt R, Thiel E. CSF evaluation in primary CNS lymphoma patients by PCR of the CDR III IgH genes. Neurology. 2002;58(3):390-396.
40. Fischer L, Martus P, Weller M, et al. Meningeal dissemination in primary CNS lymphoma: prospective evaluation of 282 patients. Neurology. 2008;71(14):1102-1108.
41. Schroers R, Baraniskin A, Heute C, et al. Detection of free immunoglobulin light chains in cerebrospinal fluids of patients with central nervous system lymphomas. Eur J Haematol. 2010;85(3):236-242.
42. Hu Y, Xiang J, Luo X, et al. Application of cerebrospinal fluid free light chain in diagnosis of primary central nervous system lymphoma and monitoring of associated chemotherapy efficacy. Clin Chim Acta. 2022;533:48-52.

43. Roy S, Josephson SA, Fridlyand J, et al. Protein biomarker identification in the CSF of patients with CNS lymphoma. J Clin Oncol. 2008;26(1):96-105.
44. Sasayama T, Nakamizo S, Nishihara M, et al. Cerebrospinal fluid interleukin-10 is a potentially useful biomarker in immunocompetent primary central nervous system lymphoma (PCNSL). Neuro Oncol. 2012;14(3):368-380.
45. Rubenstein JL, Wong VS, Kadoch C, et al. CXCL13 plus interleukin 10 is highly specific for the diagnosis of CNS lymphoma. Blood. 2013;121(23):4740-4748.
46. Sasagawa Y, Akai T, Tachibana O, Iizuka H. Diagnostic value of interleukin-10 in cerebrospinal fluid for diffuse large B-cell lymphoma of the central nervous system. J Neurooncol. 2015;121(1):177-183.
47. Nguyen-Them L, Costopoulos M, Tanguy ML, et al. The CSF IL-10 concentration is an effective diagnostic marker in immunocompetent primary CNS lymphoma and a potential prognostic biomarker in treatment-responsive patients. Eur J Cancer 2016;61:69-76.
48. Song Y, Zhang W, Zhang L, et al. Cerebrospinal Fluid IL-10 and IL-10/IL-6 as Accurate Diagnostic Biomarkers for Primary Central Nervous System Large B-cell Lymphoma. Sci Rep.2016;6:38671.
49. Mabray MC, Barajas RF, Villanueva-Meyer JE, et al. The Combined Performance of ADC, CSF CXC Chemokine Ligand 13, and CSF Interleukin 10 in the Diagnosis of Central Nervous System Lymphoma. AJNR Am J Neuroradiol. 2016;37(1):74-79.
50. Shao J, Chen K, Li Q, et al. High Level of IL-10 in Cerebrospinal Fluid is Specific for Diagnosis of Primary Central Nervous System Lymphoma. Cancer Manag Res. 2020;12:6261-6268.
51. Geng M, Song Y, Xiao H, et al. Clinical significance of interleukin-10 concentration in the cerebrospinal fluid of patients with primary central nervous system lymphoma. Oncol Lett. 2021;21(1):2.
52. Ungureanu A, Le Garff-Tavernier M, Costopoulos M, et al. CSF interleukin 6 is a useful marker to distinguish pseudotumoral CNS inflammatory diseases from primary CNS lymphoma. J Neurol. 2021;268(8):2890-2894.
53. Carbonell D, Mahajan S, Chee SP, et al. Consensus Recommendations for the Diagnosis of Vitreoretinal Lymphoma. Ocul Immunol Inflamm. 2021;29(3):507-520.

54. Strehlow F, Bauer S, Martus P, et al. Osteopontin in cerebrospinal fluid as diagnostic biomarker for central nervous system lymphoma. J Neurooncol. 2016;129(1):165-171.
55. Thaler FS, Laurent SA, Huber M, et al. Soluble TACI and soluble BCMA as biomarkers in primary central nervous system lymphoma. Neuro Oncol. 2017;19(12):1618-1627.
56. Mulazzani M, Huber M, Borchard S, et al. APRIL and BAFF: novel biomarkers for central nervous system lymphoma. J Hematol Oncol. 2019;12(1):102.
57. Lim KH, Yang Y, Staudt LM. Pathogenetic importance and therapeutic implications of NF-κB in lymphoid malignancies. Immunol Rev. 2012;246(1):359-378.
58. Montesinos-Rongen M, Godlewska E, Brunn A, Wiestler OD, Siebert R, Deckert M. Activating L265P mutations of the MYD88 gene are common in primary central nervous system lymphoma. Acta Neuropathol. 2011;122(6):791-792.
59. Poulain S, Boyle EM, Tricot S, et al. Absence of CXCR4 mutations but high incidence of double mutant in CD79A/B and MYD88 in primary central nervous system lymphoma. Br J Haematol. 2015;170(2):285-287.
60. Fukumura K, Kawazu M, Kojima S, et al. Genomic characterization of primary central nervous system lymphoma. Acta Neuropathol. 2016;131(6):865-875.
61. Nakamura T, Tateishi K, Niwa T, et al. Recurrent mutations of CD79B and MYD88 are the hallmark of primary central nervous system lymphomas. Neuropathol Appl Neurobiol. 2016;42(3):279-290.
62. Grommes C, Pastore A, Palaskas N, et al. Ibrutinib Unmasks Critical Role of Bruton Tyrosine Kinase in Primary CNS Lymphoma. Cancer Discov. 2017;7(9):1018-1029.
63. Ferreri AJM, Calimeri T, Lopedote P, et al. MYD88 L265P mutation and interleukin-10 detection in cerebrospinal fluid are highly specific discriminating markers in patients with primary central nervous system lymphoma: results from a prospective study. Br J Haematol. 2021;193(3):497-505.
64. Varettoni M, Arcaini L, Zibellini S, et al. Prevalence and clinical significance of the MYD88 (L265P) somatic mutation in Waldenstrom's macroglobulinemia and related lymphoid neoplasms. Blood. 2013;121(13):2522-2528.

65. Hattori K, Sakata-Yanagimoto M, Suehara Y, et al. Clinical significance of disease-specific MYD88 mutations in circulating DNA in primary central nervous system lymphoma. Cancer Sci. 2018;109(1):225-230.
66. Montesinos-Rongen M, Brunn A, Tuchscherer A, et al. Analysis of Driver Mutational Hot Spots in Blood-Derived Cell-Free DNA of Patients with Primary Central Nervous System Lymphoma Obtained before Intracerebral Biopsy. J Mol Diagn. 2020;22(10):1300-1307.
67. Rimelen V, Ahle G, Pencreach E, et al. Tumor cell-free DNA detection in CSF for primary CNS lymphoma diagnosis. Acta Neuropathol Commun. 2019;7(1):43.
68. Watanabe J, Natsumeda M, Okada M, et al. High Detection Rate of MYD88 Mutations in Cerebrospinal Fluid From Patients With CNS Lymphomas. JCO Precis Oncol. 2019;3:1-13.
69. Chen F, Pang D, Guo H, et al. Clinical outcomes of newly diagnosed primary CNS lymphoma treated with ibrutinib-based combination therapy: A real-world experience of off-label ibrutinib use. Cancer Med. 2020;9(22):8676-8684.
70. Gupta M, Burns EJ, Georgantas NZ, et al. A rapid genotyping panel for detection of primary central nervous system lymphoma. Blood. 2021;138(5):382-386.
71. Yamagishi Y, Sasaki N, Nakano Y, et al. Liquid biopsy of cerebrospinal fluid for MYD88 L265P mutation is useful for diagnosis of central nervous system lymphoma. Cancer Sci. 2021;112(11):4702-4710.
72. Yamaguchi J, Ohka F, Kitano Y, et al. Rapid detection of the MYD88 L265P mutation for pre- and intra-operative diagnosis of primary central nervous system lymphoma. Cancer Sci. 2023;114(6):2544-2551.
73. Bravetti C, Degaud M, Armand M, et al. Combining MYD88 L265P mutation detection and clonality determination on CSF cellular and cell-free DNA improves diagnosis of primary CNS lymphoma. Br J Haematol. 2023;201(6):1088-1096.
74. Bonzheim I, Giese S, Deuter C, et al. High frequency of MYD88 mutations in vitreoretinal B-cell lymphoma: a valuable tool to improve diagnostic yield of vitreous aspirates. Blood. 2015;126(1):76-79.

75. Miserocchi E, Ferreri AJM, Giuffrè C, et al. MYD88 L265P mutation detection in the aqueous humor of patients with vitreoretinal lymphoma. Retina. 2019;39(4):679-684.
76. Fontanilles M, Marguet F, Bohers É, et al. Non-invasive detection of somatic mutations using next-generation sequencing in primary central nervous system lymphoma. Oncotarget. 2017;8(29):48157-48168.
77. Yoon SE, Kim YJ, Shim JH, et al. Plasma Circulating Tumor DNA in Patients with Primary Central Nervous System Lymphoma. Cancer Res Treat. 2022;54(2):597-612.
78. Mutter JA, Alig SK, Esfahani MS, et al. Circulating Tumor DNA Profiling for Detection, Risk Stratification, and Classification of Brain Lymphomas. J Clin Oncol. 2023;41(9):1684-1694.
79. Hiemcke-Jiwa LS, Leguit RJ, Snijders TJ, et al. Molecular analysis in liquid biopsies for diagnostics of primary central nervous system lymphoma: Review of literature and future opportunities. Crit Rev Oncol Hematol. 2018;127:56-65.
80. Grommes C, Tang SS, Wolfe J, et al. Phase 1b trial of an ibrutinib-based combination therapy in recurrent/refractory CNS lymphoma. Blood. 2019;133(5):436-445.
81. Baraniskin A, Kuhnhenn J, Schlegel U, et al. Identification of microRNAs in the cerebrospinal fluid as marker for primary diffuse large B-cell lymphoma of the central nervous system. Blood. 2011;117(11):3140-3146.
82. Baraniskin A, Kuhnhenn J, Schlegel U, et al. MicroRNAs in cerebrospinal fluid as biomarker for disease course monitoring in primary central nervous system lymphoma. J Neurooncol. 2012;109(2):239-244.
83. Zajdel M, Rymkiewicz G, Sromek M, et al. Tumor and Cerebrospinal Fluid microRNAs in Primary Central Nervous System Lymphomas. Cancers (Basel). 2019;11(11):1647.
84. Mao X, Sun Y, Tang J. Serum miR-21 is a diagnostic and prognostic marker of primary central nervous system lymphoma. Neurol Sci. 2014;35(2):233-238.
85. Takashima Y, Kawaguchi A, Iwadate Y, et al. miR-101, miR-548b, miR-554, and miR-1202 are reliable prognosis predictors of the miRNAs associated with cancer immunity in primary central nervous system lymphoma. PLoS One. 2020;15(2):e0229577.

86. Si PP, Zhou XH, Qu ZZ. Identification of Serum miRNAs as Effective Diagnostic Biomarkers for Distinguishing Primary Central Nervous System Lymphoma from Glioma. J Immunol Res. 2022;2022:5052609.
87. Nakamura M, Sakaki T, Hashimoto H, et al. Frequent alterations of the p14(ARF) and p16(INK4a) genes in primary central nervous system lymphomas. Cancer research. 2001;61(17):6335-6339.
88. Richter J, Ammerpohl O, Martín-Subero JI, et al. Array-based DNA methylation profiling of primary lymphomas of the central nervous system. BMC Cancer. 2009;9(1):455.
89. Pirosa MC, Borchmann S, Jardin F, Gaidano G, Rossi D. Controversies in the Interpretation of Liquid Biopsy Data in Lymphoma. HemaSphere. 2022;6(6):e727.
90. Chu LC, Eberhart CG, Grossman SA, Herman JG. Epigenetic silencing of multiple genes in primary CNS lymphoma. International Journal of Cancer. 2006;119(10):2487-2491.
91. Ferreri AJ, Dell'Oro S, Capello D, et al. Aberrant methylation in the promoter region of the reduced folate carrier gene is a potential mechanism of resistance to methotrexate in primary central nervous system lymphomas. Br J Haematol. 2004;126(5):657-664.
92. Cobbers JM, Wolter M, Reifenberger J, et al. Frequent inactivation of CDKN2A and rare mutation of TP53 in PCNSL. Brain Pathol. 1998;8(2):263-276.
93. Gonzalez-Gomez P, Bello MJ, Arjona D, et al. CpG island methylation of tumor-related genes in three primary central nervous system lymphomas in immunocompetent patients. Cancer Genet Cytogenet. 2003;142(1):21-24.
94. Downs BM, Ding W, Cope LM, et al. Methylated markers accurately distinguish primary central nervous system lymphomas (PCNSL) from other CNS tumors. Clinical Epigenetics. 2021;13(1):104.
95. Levenson VV. DNA methylation as a universal biomarker. Expert Rev Mol Diagn. 2010;10(4):481-488.
96. van Dongen J, Nivard MG, Willemsen G, et al. Genetic and environmental influences interact with age and sex in shaping the human methylome. Nature communications. 2016;7:11115.
97. Vaz M, Hwang SY, Kagiampakis I, et al. Chronic Cigarette Smoke-Induced Epigenomic Changes Precede Sensitization of Bronchial Epithelial Cells to

Single-Step Transformation by KRAS Mutations. Cancer cell. 2017;32(3):360-376.

98. Alcaide M, Cheung M, Hillman J, et al. Evaluating the quantity, quality and size distribution of cell-free DNA by multiplex droplet digital PCR. Scientific reports. 2020;10(1):12564.
99. Wang Q, Gu L, Adey A, et al. Tagmentation-based whole-genome bisulfite sequencing. Nature Protocols. 2013;8(10):2022-2032.
100. Brinkman AB, Simmer F, Ma K, Kaan A, Zhu J, Stunnenberg HG. Whole-genome DNA methylation profiling using MethylCap-seq. Methods. 2010;52(3):232-236.
101. Maunakea AK, Nagarajan RP, Bilenky M, et al. Conserved role of intragenic DNA methylation in regulating alternative promoters. Nature. 2010;466(7303):253-257.
102. Gu H, Smith ZD, Bock C, Boyle P, Gnirke A, Meissner A. Preparation of reduced representation bisulfite sequencing libraries for genome-scale DNA methylation profiling. Nature Protocols. 2011;6(4):468-481.
103. Shen SY, Burgener JM, Bratman SV, De Carvalho DD. Preparation of cfMeDIP-seq libraries for methylome profiling of plasma cell-free DNA. Nature Protocols. 2019;14(10):2749-2780.
104. Zuccato JA, Patil V, Mansouri S, et al. Cerebrospinal fluid methylome-based liquid biopsies for accurate malignant brain neoplasm classification. Neuro Oncol. 2023;25(8):1452-1460.
105. Cwynarski K, Cummin T, Osborne W, et al. Management of secondary central nervous system lymphoma. Br J Haematol. 2023;200(2):160-169.
106. Gleeson M, Counsell N, Cunningham D, et al. Central nervous system relapse of diffuse large B-cell lymphoma in the rituximab era: results of the UK NCRI R-CHOP-14 versus 21 trial. Ann Oncol. 2017;28(10):2511-2516.
107. Wight JC, Yue M, Keane C, et al. Outcomes of synchronous systemic and central nervous system (CNS) involvement of diffuse large B-cell lymphoma are dictated by the CNS disease: a collaborative study of the Australasian Lymphoma Alliance. Br J Haematol. 2019;187(2):174-184.
108. Bromberg JE, Doorduijn JK, Illerhaus G, et al. Central nervous system recurrence of systemic lymphoma in the era of stem cell transplantation--an

International Primary Central Nervous System Lymphoma Study Group project. Haematologica. 2013;98(5):808-813.

109. Omuro A, Correa DD, DeAngelis LM, et al. R-MPV followed by high-dose chemotherapy with TBC and autologous stem-cell transplant for newly diagnosed primary CNS lymphoma. Blood. 2015;125(9):1403-1410.
110. Canovi S, Campioli D. Accuracy of flow cytometry and cytomorphology for the diagnosis of meningeal involvement in lymphoid neoplasms: A systematic review. Diagn Cytopathol. 2016;44(10):841-856.
111. Houillier C, Taillandier L, Dureau S, et al. Radiotherapy or Autologous Stem-Cell Transplantation for Primary CNS Lymphoma in Patients 60 Years of Age and Younger: Results of the Intergroup ANOCEF-GOELAMS Randomized Phase II PRECIS Study. J Clin Oncol. 2019;37(10):823-833.
112. Baraniskin A, Schroers R. Liquid Biopsy and Other Non-Invasive Diagnostic Measures in PCNSL. Cancers (Basel). 2021;13(11):2665.
113. Krsmanovic P, Mocikova H, Chramostova K, et al. Circulating microRNAs in Cerebrospinal Fluid and Plasma: Sensitive Tool for Detection of Secondary CNS Involvement, Monitoring of Therapy and Prediction of CNS Relapse in Aggressive B-NHL Lymphomas. Cancers (Basel). 2022;14(9):2305.
114. Hayashita Y, Osada H, Tatematsu Y, et al. A polycistronic microRNA cluster, miR-17-92, is overexpressed in human lung cancers and enhances cell proliferation. Cancer Res. 2005;65(21):9628-3962.
115. Malzkorn B, Wolter M, Liesenberg F, et al. Identification and functional characterization of microRNAs involved in the malignant progression of gliomas. Brain Pathol. 2010;20(3):539-550.
116. Kim K, Chadalapaka G, Lee SO, et al. Identification of oncogenic microRNA-17-92/ZBTB4/specificity protein axis in breast cancer. Oncogene. 2012;31(8):1034-1044.
117. Watanabe J, Natsumeda M, Kanemaru Y, et al. Comparison of circulating tumor DNA between body fluids in patients with primary central nervous system lymphoma. Leuk Lymphoma. 2019;60(14):3587-3589.
118. Bobillo S, Crespo M, Escudero L, et al. Cell free circulating tumor DNA in cerebrospinal fluid detects and monitors central nervous system involvement of B-cell lymphomas. Haematologica. 2021;106(2):513-521.

119. Olszewski AJ, Chorzalska AD, Petersen M, et al. Detection of clonotypic DNA in the cerebrospinal fluid as a marker of central nervous system invasion in lymphoma. Blood Adv. 2021;5(24):5525-5535.
120. Chabon JJ, Hamilton EG, Kurtz DM, et al. Integrating genomic features for non-invasive early lung cancer detection. Nature. 2020;580(7802):245-251.
121. Chan HT, Chin YM, Nakamura Y, Low SK. Clonal Hematopoiesis in Liquid Biopsy: From Biological Noise to Valuable Clinical Implications. Cancers (Basel). 2020;12(8).
122. Ernerudh J, Olsson T, Berlin G, von Schenck H. Cerebrospinal fluid immunoglobulins and beta 2-microglobulin in lymphoproliferative and other neoplastic diseases of the central nervous system. Arch Neurol. 1987;44(9):915-20.
123. Kersten MJ, Evers LM, Dellemijn PL, et al. Elevation of cerebrospinal fluid soluble CD27 levels in patients with meningeal localization of lymphoid malignancies. Blood. 1996;87(5):1985-1989.
124. Hildebrandt B, Müller C, Pezzutto A, Daniel P, Dörken B, Scholz C. Assessment of free light chains in the cerebrospinal fluid of patients with lymphomatous meningitis - a pilot study. BMC Cancer. 2007;7:185.
125. Muñiz C, Martín-Martín L, López A, et al. Contribution of cerebrospinal fluid sCD19 levels to the detection of CNS lymphoma and its impact on disease outcome. Blood. 2014;123(12):1864-1869.
126. Villa D, Connors JM, Shenkier TN, Gascoyne RD, Sehn LH, Savage KJ. Incidence and risk factors for central nervous system relapse in patients with diffuse large B-cell lymphoma: the impact of the addition of rituximab to CHOP chemotherapy. Ann Oncol. 2010;21(5):1046-1052.
127. Schmitz N, Zeynalova S, Nickelsen M, et al. CNS International Prognostic Index: A Risk Model for CNS Relapse in Patients With Diffuse Large B-Cell Lymphoma Treated With R-CHOP. J Clin Oncol. 2016;34(26):3150-3156.
128. Savage KJ. Secondary CNS relapse in diffuse large B-cell lymphoma: defining high-risk patients and optimization of prophylaxis strategies. Hematology Am Soc Hematol Educ Program. 2017;2017(1):578-586.
129. Savage KJ, Slack GW, Mottok A, Sehn LH, Villa D, Kansara R, et al. Impact of dual expression of MYC and BCL2 by immunohistochemistry on the risk of CNS relapse in DLBCL. Blood. 2016;127(18):2182-2188.

130. Chapuy B, Stewart C, Dunford AJ, Kim J, Kamburov A, Redd RA, et al. Molecular subtypes of diffuse large B cell lymphoma are associated with distinct pathogenic mechanisms and outcomes. Nat Med. 2018;24(5):679-690.
131. Schmitz R, Wright GW, Huang DW, J et al. Genetics and Pathogenesis of Diffuse Large B-Cell Lymphoma. N Engl J Med. 2018;378(15):1396-1407.
132. Klanova M, Sehn LH, Bence-Bruckler I, et al. Integration of cell of origin into the clinical CNS International Prognostic Index improves CNS relapse prediction in DLBCL. Blood. 2019;133(9):919-926.
133. Ollila TA, Kurt H, Waroich J, et al. Genomic subtypes may predict the risk of central nervous system recurrence in diffuse large B-cell lymphoma. Blood. 2021;137(8):1120-1124.
134. Puckrin R, El Darsa H, Ghosh S, Peters A, Owen C, Stewart D. Ineffectiveness of high-dose methotrexate for prevention of CNS relapse in diffuse large B-cell lymphoma. Am J Hematol. 2021;96(7):764-771.
135. Orellana-Noia VM, Reed DR, McCook AA, et al. Single-route CNS prophylaxis for aggressive non-Hodgkin lymphomas: real-world outcomes from 21 US academic institutions. Blood. 2022;139(3):413-423.
136. Wilson MR, Eyre TA, Kirkwood AA, et al. Timing of high-dose methotrexate CNS prophylaxis in DLBCL: a multicenter international analysis of 1384 patients. Blood. 2022;139(16):2499-2511.
137. Montesinos-Rongen M, Schafer E, Siebert R, Deckert M. Genes regulating the B cell receptor pathway are recurrently mutated in primary central nervous system lymphoma. Acta Neuropathol. 2012;124(6):905-906.
138. Grommes C, Nayak L, Tun HW, Batchelor TT. Introduction of novel agents in the treatment of primary CNS lymphoma. Neuro Oncol. 2019;21(3):306-313.
139. Braggio E, Van Wier S, Ojha J, et al. Genome-Wide Analysis Uncovers Novel Recurrent Alterations in Primary Central Nervous System Lymphomas. Clin Cancer Res. 2015;21(17):3986-3994.
140. Chapuy B, Roemer MG, Stewart C, et al. Targetable genetic features of primary testicular and primary central nervous system lymphomas. Blood. 2016;127(7):869-881.
141. Hernández-Verdin I, Kirasic E, Wienand K, et al. Molecular and clinical diversity in primary central nervous system lymphoma. Ann Oncol. 2023;34(2):186-199.

142. Rubenstein JL, Fridlyand J, Shen A, et al. Gene expression and angiotropism in primary CNS lymphoma. Blood. 2006;107(9):3716-3723.
143. Fischer L, Hummel M, Korfel A, Lenze D, Joehrens K, Thiel E. Differential micro-RNA expression in primary CNS and nodal diffuse large B-cell lymphomas. Neuro Oncol. 2011;13(10):1090-1098
144. Kubuki Y, Yamaji T, Hidaka T, et al. TET2 mutation in diffuse large B-cell lymphoma. J Clin Exp Hematop. 2017;56(3):145-149.
145. Zhou L, Li Q, Xu J, et al. Cerebrospinal fluid metabolic markers predict prognosis behavior of primary central nervous system lymphoma with high-dose methotrexate-based chemotherapeutic treatment. Neurooncol Adv. 2022;5(1):vdac181.
146. Rubenstein JL, Geng H, Fraser EJ, et al. Phase 1 investigation of lenalidomide/rituximab plus outcomes of lenalidomide maintenance in relapsed CNS lymphoma. Blood Adv. 2018;2(13):1595-1607.
147. Cheng CL, Yao CY, Huang PH, et al. Cerebrospinal fluid soluble programmed death-ligand 1 is a useful prognostic biomarker in primary central nervous system lymphoma. Br J Haematol. 2023;201(1):75-85.
148. Schmitt MW, Kennedy SR, Salk JJ, Fox EJ, Hiatt JB, Loeb LA. Detection of ultra-rare mutations by next-generation sequencing. Proc Natl Acad Sci U S A. 2012;109(36):14508-14513.
149. Kennedy SR, Schmitt MW, Fox EJ, et al. Detecting ultralow-frequency mutations by Duplex Sequencing. Nat Protoc. 2014;9(11):2586-606.
150. Newman AM, Lovejoy AF, Klass DM, et al. Integrated digital error suppression for improved detection of circulating tumor DNA. Nat Biotechnol. 2016;34(5):547-555.
151. Kurtz DM, Soo J, Co Ting Keh L, et al. Enhanced detection of minimal residual disease by targeted sequencing of phased variants in circulating tumor DNA. Nat Biotechnol. 2021;39(12):1537-1547.
152. Hoang-Xuan K, Deckert M, Ferreri AJM, et al. European Association of Neuro-Oncology (EANO) guidelines for treatment of primary central nervous system lymphoma (PCNSL). Neuro Oncol. 2023;25(1):37-53.
153. Rincon-Torroella J, Khela H, Bettegowda A, Bettegowda C. Biomarkers and focused ultrasound: the future of liquid biopsy for brain tumor patients. J Neurooncol. 2022;156(1):33-48.

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Conflict of Interest

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- *AB*: I have no conflicts of interest to declare.
- *LB*: I have no disclosures of conflict of interests.
- *JECB*: I have no relevant conflicts of interest.
- AJMF: has been the chairman of the PAMINA trial, which supports the use of MYD88L265P and IL-10 as diagnostic markers in PCNSL patients.
- *CG*: Consultation: Kite, BTG, ONO, Roche.
- *KHX*: No COI to declare.
- *JK*: I do not have any conflicts of interest to report. – Department of Medicine I, Medical Center – University of Freiburg, Faculty of Medicine, University of Freiburg, Freiburg, Germany.
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Author Contribution

L. Nayak, C. Bettegowda, F. Scherer and N. Galldiks wrote specific sub-sections.

R. Soffietti coordinated the writing review of the review and editing of the manuscript.

All other authors took part in discussions, amendments and final approval.

Table 1. Identification of lymphoma cells by CSF cytopathology and flow cytometry: comparative analyses

Reference	Number of patients	CSF cytology positive	CSF flow cytometry positive	Sensitivity/ Specificity of flow cytometry using cytology as gold standard
Finn et al,1998 [30]	36	7 (19%)	9 (25%)	86%/83%
French et al, 2000 [31]	35	6 (17%)	7 (20%)	-
Hegde et al, 2005 [32]	51 9	1 (2%) 1 (11%)	11(22%) 3 (33%)	100%/80% 100%/75%
Quijano et al, 2009 [33]	123	7 (6%)	27 (22%)	88%/85%
Kiewe et al, 2010 [34]	69	7/63 (11%)	1/32 (3%)	24%/100%
Schroers et al, 2010 [35]	37	7 (19%)	11 (30%)	86%/83%
Stacchini et al, 2012 [37]	62	10 (16%)	15 (24%)	100%/90%
Benevolo et al, 2012 [36]	174	7 (4%)	18 (10%)	100%/95%

Table 2. Interleukin-10, interleukin-6 and CXCL13 in the diagnosis of CNS lymphoma

Reference	N, Total (CNSL)	Protein biomarker	Median level (pg/mL)	Cut-off level (pg/mL)	Sensitivity	Specificity
Sasayama et al, 2012 [44]	66 (26 PCNSL, 40 other BT) Prospective 5/24	IL-10 IL-6 IL-10 IL-6	27 5.4 42 6.8	9.5	71%	100%
Rubenstein et al, 2013 [45]	220 (83 CNSL, 147 others)	IL10 CXCL13 IL10 or CXCL13 IL10 + CXCL13	57-1003 539-5926	16.15 90 23 or 116	65.4% 69.9% 84.2% 50%	92.6% 92.7% 90.5% 99.3%
Sasagawa et al, 2015 [46]	19 CNSL, 26 others	IL10	28	3	94.7%	100%
Nguyen-Them et al, 2016 [47]	152 (112 PCNSL & PVRL, 40 others)	IL10		4	88.6%	88.9%
Song et al, 2016 [48]	22 PCNSL, 80 others	IL10 IL10/IL6		8.2 0.72	95.5% 95.5%	96.1% 100%
Mabray et al, 2016 [49]	87 (43 CNL, 44 others)	IL10 CXCL13		20.6 262.82	95% 95%	95% 95%
Shao et al, 2020 [50]	108 (66 PCNSL, 42 other BTs)	IL10 IL10/IL6	58.2 24.3	8.3 1.6	59% 66%	98% 91%
Ungureanu et al, 2021 [52]	43 (28 PCNSL, 15 others)	IL10 IL10/IL6	36 3.89	1	89%	100%
Geng et al, 2021 [51]	91 (38 PCNSL, 53 other BTs)	IL10		10.13	97.4%	100%

Table 3. Liquid biopsy in PCNSL: studies on MYD88 LP265P mutation at diagnosis

Reference	Patients	Biofluid	Method	Sensitivity	Specificity	Type of comparison
Hattori et al, 2018 [65]	14	Serum	ddPCR	57.1%	-	no controls
Hiemcke-Jiwa et al, 2018 [79]	3	CSF	ddPCR	1/3	100%	comparison with 9 LPL, 3 SCNSL and 20 non lymphomatous lesions
Rimelen et al, 2019 [67]	11	CSF	ddPCR	86%	100%	10 non lymphomatous lesions
Watanabe et al, 2019 [68]	21	CSF	ddPCR	80.9%	-	no controls
Montesinos-Rongen et al, 2020 [66]	27	Blood	ddPCR	4%	low	with 6 healthy controls and 4 Waldenstrom disease
Ferreri et al, 2021 [63]	36	CSF	TaqManPCR	72%	-	with 106 neurological controls with 44 systemic DLBCL
Gupta et al, 2021 [70]	117 (miscellanea)	CSF plasma	TetRS (MYD88 vs TERT)	65.8%	100%	
Yamagishi et al, 2021 [71]	42	CSF	ddPCR	92.2%	100%	with 1 control

Table 4. Liquid biopsy in PCNSL: studies on ctDNA at diagnosis.

Reference	Patients	Biofluid	Method	Sensitivity	Specificity	Type of comparison
Fontanilles et al, 2017 [76]	25	Plasma	NGS	24%	100%	with 25 solid cancers
Chen et al, 2020 [69]	11	CSF	NGS	63.5%	-	no controls
Yoon et al, 2022 [77]	42	Plasma	NGS	27%	-	no controls
Mutter et al, 2023 [78]	92	plasma CSF	deep sequencing	Plasma 78%% CSF 100%	Plasma 96% CSF 97%	with 44 other CNS malignancies or inflammatory diseases and 24 healthy controls

Table 5. Studies investigating biomarkers for the diagnosis of SCNSL.

Publication	PMID	Analyte	Material	Parameter	SCNSL type	SCNSL entity	No. SCNSL[a]	No. controls (Non-CNS-lymphoma)[b]	No. controls (other)[c]	Tumor-informed vs. tumor-agnostic	Measure / threshold	Sensitivity	Specificity
Hildebrandt et al. 2007 [124]	17915026*	protein	CSF	kappa/lambda FLC ratio	TN-SCNSL	CLL, LPL, DLBCL	5	12	0	tumor-agnostic	Upper normal serum reference	100%	67%
Schroers et al. 2010 [41]	20528903*	protein	CSF	kappa/lambda FLC ratio	RI-SCNSL	DLBCL	4	0	14	tumor-agnostic	3.0	50%	100%
Ernerudh et al. 1987 [122]	3304227	protein	CSF	beta2-MG	TN-SCNSL	ALL, CLL, B-NHL	14	12	0	tumor-agnostic	1.9 mg/L	71%	67%
Rubenstein et al. 2013 [45]	23570798*	protein	CSF	IL10	TN-SCNSL, RC-SCNSL	DLBCL, BL, MZL	23	0	137	tumor-agnostic	16.5 pg/mL	61%	94%
				CXCL13							90 pg/mL	70%	95%
				CXCL13 and/or IL10							90 pg/mL and/or 16.5 pg/mL	74%	99%
Sasagawa et al. 2015 [46]	25258254*	protein	CSF	IL10	no information provided	DLBCL	4	0	26	tumor-agnostic	3 pg/mL	100%	100%
				IL10/IL6 ratio							2.2	75%	96%
				sIL2-R							60.4	100%	85%

											U/mL		
				beta2-MG							2.4 mg/L	75%	89%
Muñiz et al. 2014 [125]	24501214	protein	CSF	sCD19	TN-SCNSL	DLBCL, BL	21[$]	92	0	tumor-agnostic	1.18 ng/mL	NA	NA
Kersten et al. 1996 [123]	8634448	protein	CSF	sCD27	TN-SCNSL	ALL, B-NHL (group III)	45^	70	82	tumor-agnostic	10 U/mL	100%	82%
				beta2-MG							1.6 mg/L	81%	68%
Krsmanovic et al. 2022 [113]	35565434	miRNA	CSF	Combination of various microRNAs: oncomiR index; RT-qPCR	TN-SCNSL RI-SCNSL RC-SCNSL	DLBCL, MCL, BL, B-NHL NOS	54	108	22	tumor-agnostic	Various thresholds	DLBCL 91%, MCL 88%, BL 100%, B-NHL NOS 100%	DLBCL 90%, MCL 93%, BL 100%, B-NHL NOS 89%
			Plasma								Various thresholds	DLBCL 83%, MCL 79%, BL 100%, B-NHL NOS 100%	DLBCL 78%, MCL 100%, BL 100%, B-NHL NOS 67%
Watanabe et al. 2019 [68]	35100686*	cfDNA	CSF	*MYD88* L265P ddPCR	RI-SCNSL	DLBCL	5	0	0	tumor-agnostic	0.1% AF	60%	NA
										tumor-informed	0.1% AF	100%	NA
Bobillo et al. 2021 [118]	32079701	ctDNA	CSF	ctDNA mut. ddPCR	RI-SCNSL RC-SCNSL	DLBCL, LPL, MCL	6	12	10	tumor-informed	Unclear	83%	100%
Olszewski et al. 2021 [119]	34551072	ctDNA	CSF	*Ig* clonotype NGS	TN-SCNSL	DLBCL, DHL, BL	5	0	0	tumor-agnostic	Unclear	100%	NA
Mutter et al. 2023 [78]	36542815*	ctDNA	CSF	ctDNA mut Targeted NGS/PhasED-Seq	RI-SCNSL	DLBCL	4	0	24	tumor-informed	Threshold based on MC framework	100%	97%

			Plasma				14				Threshold based on MC framework	86%	96%

*These studies primarily focus on PCNSL but cover SCNSL as a sub-cohort; yet this table is restricted to SCNSL patients only. Studies that include SNCSL but do not allow to differentiate between primary and secondary CNSL, those focusing on children, or those investigating only 1-2 lymphoma patients were not considered (2, 3, 4, 5, 6, 7). Only those studies are listed for which dedicated information on the SCNSL cohort is available. [a] Number of SCNSL patients included in the study; [b] Control cohort of patients with systemic lymphoid malignancy and no sign of overt CNS involvement; [c] Control cohort of patients with other neurological disorders and no sign of lymphoid malignancy or healthy subjects. n, number of patients; FLC, free light chain; MG, microglobulin; RT-qPCR, real-time quantitative PCR; NGS, next-generation sequencing; PhasED-Seq, phased variant enrichment and detection sequencing; SCNSL, secondary CNS lymphoma; TN, treatment-naïve; RI, isolated CNS relapse: RC, CNS and systemic relapse; CLL, chronic lymphocytic leukemia; LPL, lymphoplasmocytic lymphoma; DLBCL, diffuse large B cell lymphoma; NHL, Non-Hodgkin lymphoma; ALL, acute lymphocytic leukemia; MCL, mantle cell lymphoma; MZL, marginal zone lymphoma; BL, Burkitt lymphoma; PTLD, post-transplantation lymphoproliferative disorder; NOS, not otherwise specified; DHL, double-hit lymphoma; ng, nanogram; pg, picogram; mg, milligram; mL, milliliter; L, liter; U, units; AF, allele frequency; mut, mutations; MC, monte carlo; *Ig*, immunoglobulin; NA, not assessed. Values for sensitivity and specificity were rounded. [$]Patients with positive flow cytometry results only. ^ Group III includes 18 patients with ALL and 27 with NHL. The test performance is reported for the whole group, the entities have not been considered separately.

Table 6. Suggested clinical applications of liquid biopsy for the diagnosis and monitoring in CNS lymphomas

Applications for CNS lymphomas diagnosis
• Diagnosis of PCNSL in high risk situations (elderly or frail patients, deep locations).
• Differential diagnosis of PCNSL versus malignant glioma to guide the surgical strategy (biopsy vs extensive resection) in favorable locations.
• Diagnosis of PCNSL in steroid-responsive patients to avoid unrevealing biopsies.
• Diagnosis of occult CNS involvement in systemic lymphomas.
Applications for CNS lymphomas monitoring
• Definition of minimal residual disease.
• Early indication of tumor response or recurrence following cytotoxic or targeted agents.
• Predictive value of MYD88 L265P mutation.
• Identification of patients at low versus high risk for resistance or disease progression after first-line treatments → early adjustment of current therapy or shift to investigational drugs.

Table 7. Ongoing clinical trials on primary CNS lymphomas including liquid biopsy.

NCT	Phase	N		Primary endpoint	Secondary endpoints
NCT05600660	2 - single arm	28	Orelabrutinib + Rituximab+ Methotrexate	Objective response rate	Progression free survival, Treatment-related adverse events, **ctDNA mutation and mean ctDNA concentration in serum and CSF, The levels of cytokine concentration in serum and CSF (ELISA)**
NCT05135858	1 (3+3 escalation design)	18	Glucarpidase + methotrexate	Dose limiting toxicity	Adverse events, overall response rate, neurocognition assessment, quality of life, **mean of dosage of CSF IL-10**
NCT05036577	1 (dose escalating study)	15	Orelabrutinib + Rituximab + Methotrexate + Dexamethasone	Maximum tolerated dose	Objective response rate, time to response, progression free survival, overall survival, **cytokine in the CSF (flow cytometry), the levels of ctDNA in the CSF**
NCT05625594	1	20	Intraventricular infusion of CD19CAR-CD28-CD3zeta-EGFRt-expressing Tcm-enriched T-lymphocytes	Incidence of adverse events, disease response rate	CAR- T and endogenous T cell levels and phenotype in peripheral blood and CSF, Progression free survival ,overall survival, **cytokine levels in peripheral blood and CSF,**
NCT04401774	2	25	Nivolumab	Incidence od adverse events, **cfDNA conversion rate in CSF**	---
NCT05036564	Not applicable	70	---	**to design a panel of "core"**	

				genetic alterations by sequencing CSF DNA in patients with confirmed or suspicious PCNSL with the aim to improve diagnostic sensitivity, response assessment and monitoring early CNS relapse in routine practice; association between recurrent genetic alterations and PCNSL diagnosis or relapse; ssociation between recurrent genetic alterations and residual enhanced and not-enhanced images at the MRI	
NCT04961515	1/2	48	Orelabrutinib + Sintilimab	Tolerable dose, objective response rate	Progression free survival, overall survival, duration of response**, ctDNA mutation (NGS) and mean ctDNA concentration in serum and CSF, the levels of cytokine concentration in serum and CSF (ELISA)**
NCT05117814	Prospective	20	Zanubrutinib	Overall response rate	Time to response, duration of response, concentration of zanunbrutinib in CSF and plasma, progression free survival, adverse events, overall survival, **ctDNA in**

					CSF
NCT05828628	Prospective	36	A prospective imaging and translational tissue study in CNS lymphoma to enable further disease characterisation and the development of potential predictive and prognostic biomarkers	**Plasma ctDNA detection**	**Plasma ctDNA detection in peripheral blood, urine, and CSF at baseline, at end of treatment, at disease progression and correlation with radiological data**
NCT04481815	2	240	Rituximab + Lenalidomide + Methotrexate versus Rituximab + Methotrexate	2-year progression-free survival	Objective response rate, overall survival, progression-free survival, adverse events, **next generation sequencing of serum and tissue samples to identify ctDNA and tissue biomarkers before and after treatment**
NCT05021770	1/2	29	Orelabrutinib + Thiotepa	Maximum tolerated dose, objective response rate	Duration of overall response rate, progression freee survival, overall survival, toxicity profile, **ctDNA in CSF (NGS)**
NCT05698147	1/2	30	Selinexor + Rituximab + Methotrexate	maximum tolerated dose, Recommended Phase 2 Dose, objective response rate	Duration of response, progession free survival, overall response rate, adverse events, **concentration of interleukin-10, interleukin-6, CXCL-13 cytokine in CSF, ctDNA in the CSF (NGS)**

Table 8. Ongoing clinical trials on primary CNS lymphomas including liquid biopsy.

NCT	Phase	N		Primary endpoint	Secondary endpoints
NCT05600660	2 - single arm	28	Orelabrutinib + Rituximab+ Methotrexate	Objective response rate	Progression free survival, Treatment-related adverse events, **ctDNA mutation and mean ctDNA concentration in serum and CSF, The levels of cytokine concentration in serum and CSF (ELISA)**
NCT05135858	1 (3+3 escalation design)	18	Glucarpidase + methotrexate	Dose limiting toxicity	Adverse events, overall response rate, neurocognition assessment, quality of life, **mean of dosage of CSF IL-10**
NCT05036577	1 (dose escalating study)	15	Orelabrutinib + Rituximab + Methotrexate + Dexamethasone	Maximum tolerated dose	Objective response rate, time to response, progression free survival, overall survival, **cytokine in the CSF (flow cytometry), the levels of ctDNA in the CSF**
NCT05625594	1	20	Intraventricular infusion of CD19CAR-CD28-CD3zeta-EGFRt-expressing Tcm-enriched T-lymphocytes	Incidence of adverse events, disease response rate	CAR- T and endogenous T cell levels and phenotype in peripheral blood and CSF, Progression free survival ,overall survival, **cytokine levels in peripheral blood and CSF,**
NCT04401774	2	25	Nivolumab	Incidence od adverse events, **cfDNA conversion rate in CSF**	---
NCT05036564	Not applicable	70	---	**to design a panel of "core"**	

				genetic alterations by sequencing CSF DNA in patients with confirmed or suspicious PCNSL with the aim to improve diagnostic sensitivity, response assessment and monitoring early CNS relapse in routine practice; association between recurrent genetic alterations and PCNSL diagnosis or relapse; ssociation between recurrent genetic alterations and residual enhanced and not-enhanced images at the MRI	
NCT04961515	1/2	48	Orelabrutinib + Sintilimab	Tolerable dose, objective response rate	Progression free survival, overall survival, duration of response**, ctDNA mutation (NGS) and mean ctDNA concentration in serum and CSF, the levels of cytokine concentration in serum and CSF (ELISA)**
NCT05117814	Prospective	20	Zanubrutinib	Overall response rate	Time to response, duration of response, concentration of zanunbrutinib in CSF and plasma, progression free survival, adverse events, overall survival, **ctDNA in**

					CSF
NCT05828628	Prospective	36	A prospective imaging and translational tissue study in CNS lymphoma to enable further disease characterisation and the development of potential predictive and prognostic biomarkers	**Plasma ctDNA detection**	**Plasma ctDNA detection in peripheral blood, urine, and CSF at baseline, at end of treatment, at disease progression and correlation with radiological data**
NCT04481815	2	240	Rituximab + Lenalidomide + Methotrexate versus Rituximab + Methotrexate	2-year progression-free survival	Objective response rate, overall survival, progression-free survival, adverse events, **next generation sequencing of serum and tissue samples to identify ctDNA and tissue biomarkers before and after treatment**
NCT05021770	1/2	29	Orelabrutinib + Thiotepa	Maximum tolerated dose, objective response rate	Duration of overall response rate, progression freee survival, overall survival, toxicity profile, **ctDNA in CSF (NGS)**
NCT05698147	1/2	30	Selinexor + Rituximab + Methotrexate	maximum tolerated dose, Recommended Phase 2 Dose, objective response rate	Duration of response, progession free survival, overall response rate, adverse events, **concentration of interleukin-10, interleukin-6, CXCL-13 cytokine in CSF, ctDNA in the CSF (NGS)**