UPDATE

The coming of age of liquid biopsy in neuro-oncology

Giulia Berzero,[1,2,†] Valentina Pieri,[1,†] Pietro Mortini,[2,3] Massimo Filippi[1,2,4] and Gaetano Finocchiaro[1]

[†]**These authors contributed equally to this work.**

Abstract

The clinical role of liquid biopsy in oncology is growing significantly. In gliomas and other brain tumors, targeted sequencing of cell-free DNA (cfDNA) from cerebrospinal fluid (CSF) may help differential diagnosis when surgery is not recommended and be more representative of tumor heterogeneity than surgical specimens, unveiling targetable genetic alterations. Given the invasive nature of lumbar puncture to obtain CSF, the quantitative analysis of cfDNA in plasma is a lively option for patient follow-up. Confounding factors may be represented by cfDNA variations due to concomitant pathologies (inflammatory diseases, seizures) or clonal hematopoiesis. Pilot studies suggest that methylome analysis of cfDNA from plasma and temporary opening of the blood-brain barrier by ultrasounds have the potential to overcome some of these limitations. Together with this, an increased understanding of mechanisms modulating the shedding of cfDNA by the tumor may help to decrypt the meaning of cfDNA kinetics in blood or CSF.

Author affiliations:

1Neurology Unit, IRCCS San Raffaele Scientific Institute, 20132 Milan, Italy

2 Vita-Salute San Raffaele University, 20132 Milan, Italy

3 Department of Neurosurgery and Gamma Knife Radiosurgery, IRCCS San Raffaele Scientific Institute, 20132 Milan, Italy

4 Neurorehabilitation Unit; Neurophysiology Unit; Neuroimaging Research Unit, Institute of Experimental Neurology, Division of Neuroscience, IRCCS San Raffaele Scientific Institute, 20132 Milan, Italy

Correspondence to:

Gaetano Finocchiaro, MD

Neurology Unit, IRCCS San Raffaele Scientific Institute

Via Olgettina, 60

20132, Milano

Italy

E-mail: finocchiaro.gaetano@hsr.it

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Abbreviations: AI = artificial intelligence; BBB = blood-brain barrier; cfDNA = cell-free DNA; CH = clonal hematopoiesis; CTCs = circulating tumor cells; EVs = extracellular vesicles; GBM = glioblastoma; LM = leptomeningeal metastases; MAF = mutant allelic frequencies; ML = machine learning; MRgFUS = MR-guided focused ultrasound; NETs = neutrophil extracellular traps; NFRs = Nucleosome-free DNA regions; OS = overall survival; PFS = progression-free survival; TEP = tumor-educated platelets; WGS = whole-genome sequencing

The challenges of liquid biopsy in neuro-oncology

In the late 1970s, evidence was found that cancer patients have higher levels of circulating cell-free DNA (cfDNA) in plasma than healthy subjects.[1] It was later demonstrated that part of this cfDNA is tumor-derived[2] and could be used for the noninvasive detection of cancer mutations[3,4], resulting in the term "liquid biopsy" to refer to the noninvasive assessment of tumor genetic profiles using tumor-derived nucleic acids.

In the last decade, liquid biopsies have been increasingly used for response assessment, early detection of treatment resistance, prognostic prediction, and treatment decisions, complementing or even replacing tissue biopsies in precision oncology.[5] This is confirmed by the validation of commercial assays of liquid biopsy for clinical use.[6,7] In colon cancer, tumor cfDNA-guided approaches reduced adjuvant chemotherapy use without compromising

recurrence-free survival.[8] In a prospective study of 1127 patients with non-small cell lung cancer, the detection of tumor cfDNA predicted shorter survival, therapeutic targets identified by sequencing of the cfDNA provided survival advantage, and mutations specific to cfDNA were discovered in one quarter of cases, representing subclonal resistance drivers.[9]

As a result of anatomical barriers (e.g. the blood-brain barrier, BBB) that are only partially disrupted by tumors, plasma-based liquid biopsy poses a greater challenge in neuro-oncology[10] (Figure 1). Among patients with gliomas, less than 10% had plasma-derived tumor-derived cfDNA, whereas 74-100% had CSF-derived tumor-derived cfDNA.[11] The shedding of cfDNA into the CSF was especially informative in patients with high tumor burden, progressive tumors, or tumors adjacent to ventricles.[12,13] CSF-cfDNA was more informative than plasma-cfDNA for targeted sequencing and mutation detection, identifying at least one tumor-derived genetic alteration in the majority of patients with gliomas or brain metastases.[12–19] The lumbar puncture, however, is an invasive procedure and is not performed in the presence of intracranial hypertension. External ventricular drainage can be a source of CSF when ventriculostomy is required in brain tumor patients. Its use as a source of CSF, however, is hampered by possibility of obstructions and infections that may increase the amount of cfDNA simulating tumor progression.[20,21]

Thus, in the longitudinal sampling of patients in neuro-oncology, plasma is a preferable source of cfDNA (Figure 2), as exemplified by patient follow-up with highly sensitive PCR techniques like digital PCR to detect *TERT* mutations, present in more than 60% of gliomas, with 90% specificity and 62.5% sensitivity.[22] Plasma was also the source to set-up a sensitive, immunoprecipitation-based protocol to analyze the methylome of small amounts of circulating cfDNA, enabling detection of large-scale DNA methylation changes enriched for tumor-specific patterns.[23] This approach was successfully expanded to tumors in the brain, as we will discuss below.[24]

Detecting tumor cell-free DNA

Observations of cfDNA fragment size distributions identified peaks corresponding to DNA associated with nucleosomes (~147 base pairs), suggesting that cfDNA is protected from nuclease digestion through associations with nucleosome core particles. As these associations are tissue-specific, comprehensive analysis of plasma-derived cfDNA allowed predicting

nucleosome spacing and transcription factor footprints useful to track their tissue of origin, which in healthy individuals was lymphoid and myeloid cells.[25]

Cancer patients have higher levels of cfDNA in plasma compared to healthy subjects because of the high turnover of tumor cells.[26] Necrotic and apoptotic cells shed DNA fragments in extracellular fluids including plasma and CSF, again reflecting the genetic and epigenetic features of the cell of origin.[27] Increasing sequencing depth and using corrective algorithms allow to partially compensate for the presence of limited amounts of tumor cfDNA.[28]

Pediatric brain cancers

Liquid biopsy has made considerable progress in the follow-up of pediatric patients. In medulloblastomas, low-coverage whole-genome sequencing (WGS) of CSF-derived cfDNA used to detect minimal residual disease (MRD), unveiled tumor mutations at baseline in 83% of cases with metastatic spreading of the disease.[29] During therapy, persistence of MRD in cfDNA was associated with increased risk of progression. A similar fraction of altered cfDNA in the CSF was found by targeted sequencing (MSK-IMPACT) in a cohort of pediatric patients with medulloblastoma and other brain tumors.[30] However, in another cohort including 258 patients with different diagnosis (mostly high-grade gliomas) using ultra-low pass WGS copy number enabled the detection of molecular alterations in 9/46 CSF, 3/230 plasma, and 0/153 urine samples.[31]

Diffuse midline gliomas, more frequent in childhood, are characterized by the H3K27M mutation of the H3 histone gene that has been analyzed by digital PCR in cfDNA from CSF and plasma of patients enrolled in the NCT03416530 trial.[32] Decrease of the Mutant Allelic Frequencies (MAF) in the CSF was associated with prolonged progression-free survival (PFS) ($p<0.004$), while 25% increase or more of MAF was predictive of progression in half of the patients, both in plasma and CSF.[32]

Extracellular vesicles

Machine-learning classification of plasma-derived extracellular vesicles (EVs) cargo, including immunoglobulins, revealed 95% sensitivity and 90% specificity in detecting different cancers not originating in the brain.[33] Initial evidence supports a role for EVs in the

diagnosis and clinical follow-up of gliomas.[34,35] Meningiomas release sizable amounts of EVs and their amount decreases after surgery.[36] In vitro EV DNA from meningioma cultures reflect the genetic, epigenetic, and mutational landscape of the tumor of origin.[36]

EVs from glioblastoma (GBM) patients can help predict prognosis,[37-42] mirror modifications of tumor volume before and after surgery[43], and express specific GBM markers.[44] They contained EGFRvIII[45,46] and, intriguingly, amounts of the von Willebrand factor significantly higher than in healthy controls.[47]

EVs may play a role in conditioning the GBM immune milieu. Exosomes, one type of EVs, can transport TGF-β, one major immune suppressive cytokine produced by GBM.[48] In a cohort of patients receiving a dendritic cell vaccination, IL-8 (but also TGF-β) mRNA in plasma exosomes, positively correlated with immunological response to glioma antigens.[49] On the other hand, exosomes in the CSF of GBM patients contain the LGALS9 ligand that inhibits antigen processing and presentation by dendritic cells by binding their TIM3 receptor.[50] Recent data suggest EVs-mediated interactions of GBM and natural killer cells showing that circulating immune vesicles "carry unique tumor-specific signals".[51]

Overall evidence for a prominent role of EVs for diagnosis or clinical follow up of brain tumors in the peripheral blood or the CSF requires more data. For instance, qalectin-3 binding protein in blood plasma and plasma-derived EVs was at significantly higher levels in GBM patients than in healthy controls, but detection accuracy in predicting patient mortality was considerably higher in plasma than plasma EVs (75% vs. 45%, respectively).[52]

Circulating tumor cells

The presence of circulating tumor cells (CTCs) in peripheral blood originating in primary brain tumors has been described years ago but their scarcity has limited their clinical use.[53-56] On the contrary, selection of CTCs in CSF can play a relevant role in disease staging and follow-up in patients affected by medulloblastoma[57] and leptomeningeal metastases[58] (LM). In LM, CSF-CTCs were detected in 43/95 samples and 1 CSF-CTC/mL provided a diagnostic threshold with high sensitivity and specificity.[58] A CTCs assay based on epithelial cell adhesion molecule (EpCAM) immunoflow cytometry in CSF of patients with suspected LM showed higher sensitivity than cytology (94% vs 76%).[59] In LM patients treated by proton cranio-spinal irradiation, the presence of < 53 CTCs in 3 ml of CSF was associated with

longer PFS (12 vs. 6 months; p<0.01).[60] cfDNA, however, showed higher mutation rates (43.6% vs 19.8%) and MAF (41.1% vs 13.0%; p 0.0001) than CTCs.[61]

Tracking glioma spatial heterogeneity and branched evolution

Gliomas and GBM are heterogeneous tumors composed of several clonal cell populations with different genetic and transcriptional profiles.[62] Because of this, biopsy samples obtained from a single area may not represent the entire tumor while liquid biopsies might provide a more comprehensive representation of tumor molecular profiles. In one of 13 gliomas studied by shallow WGS, molecular alterations were detected in CSF cfDNA and not in one corresponding surgical specimen.[63] In 17 gliomas, 34% of the mutations found by targeted sequencing were detected in the CSF only.[64]

Under the selective pressure of alkylating chemotherapy, GBM cells are subject to hypermutation and branched clonal evolution.[65] Since only a minor proportion of GBM patients undergo second surgery at recurrence,[66] liquid biopsies offer the chance to reassess tumor molecular profile and to monitor tumor evolution during treatment with MAF in plasma and CSF, as suggested by preliminary evidence in brainstem pediatric gliomas[67–69] (Table 1). Along the same line, longitudinal liquid biopsies of plasma might support neuroimaging in response assessment (e.g. pseudoprogression vs. true progression), a clinically relevant point raised by the liquid biopsy task-force of the RANO (Response Assessment in Neuro-Oncology) consortium[70].

Recently, evidence has emerged that liquid biopsies might also have a prognostic role, given that tumor cfDNA detection in the CSF is a strong independent predictor of reduced overall survival (OS).[15] In plasma, a first study in 42 GBM patients showed correlations between shorter progression-free survival and cfDNA higher than 13.4 ng/ml.[71] In a larger subsequent study on 62 GBM using a higher threshold (25. 2 ng/ml), higher values of cfDNA were also associated with shorter overall survival.[72] This observation was challenged by data on another cohort of GBM patients, showing correlations between cfDNA levels and disease progression but not between cfDNA levels before surgery and survival[73] (Table 1).

Identifying therapeutic targets

Although in glioma patients the number of actionable molecular alterations for targeted therapies is limited,[74] the potential for their non-invasive identification is appealing. Studies of patient cohorts of primary brain tumors showed that actionable genetic alterations - including *IDH1*, *BRAF*, *EGFR*, and *NRAS* mutations - could be identified from plasma cfDNA in 18% to 24% of patients.[75,76]

Liquid biopsies can also be considered for targeted therapy of brain metastases, which show divergence in terms of genetic profile[77,78] and immune microenvironment[79] with respect to the primary tumor. As an example, by identifying *EGFR* mutations as an intervening mechanism of resistance to EGFR inhibitors,[80,81] liquid biopsies might provide a chance for a prompt switch in therapy.

The observation that MAF in the CSF decreases with response to treatment and increases at recurrence[82,83] confirms further the potential of CSF-based liquid biopsies.

The "background noise" in plasma-based liquid biopsy

cfDNA levels can change during the day and in the presence of stress[84,85].

A confounding factor for interpreting the results of plasma liquid biopsies is represented by clonal hematopoiesis (CH), which may lead to the accumulation of non-tumoral somatic mutations in hematopoietic stem cells[86]. If interpreted as tumor-associated genetic alterations in cfDNA analyses, they could steer toward inappropriate therapeutic decisions.[86,87] CH mutations are quite common in the general population, associated with aging (9.5-18.4% beyond 70 years old), prior radiotherapy or chemotherapy, and increased risk of developing hematological malignancies and cardiovascular diseases.[87,88] They mainly include mutations in epigenetic modulators (*DNMT3A*, *TET2*, and *ASXL*), and in genes often altered in solid (*KRAS*, *GNAS*, *NRAS*, and *PIK3CA*) and hematological tumors (*JAK2*, *PPM1D*, *TP53*, *SF3B1*, *SRSF2*, *IDH1*, and *IDH2*). Because of CH, the finding of *IDH1* and *IDH2* mutations in plasma-derived cfDNA of patients with gliomas may require investigation of their origin by sequencing the DNA from white blood cells (and tumor tissue, when available).[86] The relevance of CH was demonstrated by the finding of mutations in peripheral blood but not in tumor tissue in 17 of 18 patients tested.[89] Of note, CH is more frequent after chemoradiotherapy and is associated with shorter OS.

Other factors creating background noise in neuro-oncology include venous thromboembolism,[90] inflammation,[91] sepsis[92], and trauma.[92] In the plasma of septic patients, a positive correlation was present between the levels of myeloperoxidase, an enzyme released by activated neutrophils, and the amounts of cfDNA. NETosis, a specific form of cell death in which activated neutrophils release neutrophil extracellular traps (NETs) in response to inflammation, infection, or hypoxia. Myeloperoxidase, released by activated neutrophils, was found significantly correlated to cfDNA levels in septic patients.[92] NETosis can also be the underlying cause of the increased release of cfDNA in autoimmune diseases like lupus erythematosus and in rheumatoid arthritis.[93]

Of relevance in neuro-oncology is also the observation of increased cfDNA in the blood of patients with epileptic seizures. This was signaled in 2013: the difference in cfDNA concentrations between patients and controls was limited but reached statistical significance.[94] The values however were considerably higher than expected (micrograms/ml rather than nanograms/ml), suggesting the presence of contaminating nuclear DNA. Confirmation in other patient cohorts would help to validate these observations.

Increasing the clinical translation of liquid biopsy in neuro-oncology

The large majority of tumor cfDNA does not contain tumor-defining mutations. Epigenetic profiles, on the other hand, are widely distributed in the genome[95] and their evolution is modulated by the number of cell divisions as in aging and cancer, where DNA methylation loss becomes more frequent.[96,97] The potential of methylation analysis in brain tumor diagnostics is well illustrated by the development of a DNA methylation-based classification of CNS tumors founded on a machine-learning (ML) approach.[98] The use of advanced methylation-based sequencing allowed the identification of specific methylation signatures and accurate classification of brain tumors from circulating cfDNA.[24] A similar result was replicated by another group, who developed a ML algorithm to distinguish patients with or without glioma based on a cfDNA-derived methylation signature.[99]

In vitro, epigenetic profiling was also used to characterize the DNA cargo of glioma EVs.[100] *In vivo,* EVs may be released by neuronal-like subpopulation of glioma interconnecting with the surrounding normal astrocytes and neurons through microtubes, a key strategy favoring

invasion.[101] EVs are more abundant in GBM patients than in controls and their protein cargo may allude to GBM biology.[43]

Of note, blood platelets have the ability to take up secreted RNA-containing membrane vesicles derived from glioma cells.[102] Recent data showed that tumor-educated platelet (TEP) RNA-based blood tests enable the detection of 18 cancer types. Specifically, the detection rate in GBM was 51%.[103]

As mentioned above, the BBB limits the shedding of biomarkers, such as cfDNA, from brain tumors into the bloodstream, hampering their detection by conventional assays. Transcranial MR-guided focused ultrasound (MRgFUS) can transiently open the BBB, providing an opportunity for less-invasive access to brain pathology. Meng et al showed first-in-human proof-of-concept in nine GBM patients by finding that MRgFUS acutely enhances plasma cfDNA (2.6 ± 1.2-fold, $p < .01$, Wilcoxon signed-rank test).[104]

Artificial Intelligence

Artificial Intelligence (AI) and, specifically, ML shows considerable potential also for the elaboration of data gained by liquid biopsy.[105] For instance, in patients with colorectal cancer (n=72), a multimodal liquid biopsy resulting from a ML algorithm combining analysis of CTCs, exosomes and cfDNA outperformed each of the three biomarkers in predicting OS.[106] Notably, the ML-generated classifier that utilizes whole genome methylation sequencing showed highest detection sensitivity out of 10 machine-learning classifiers trained on the same samples.[107]

An "upstream" size selection (an example of cfDNA fragmentomics) can help enrich tumor cfDNA that are typically shorter than normal cfDNA fragments. By selecting *in silico* DNA fragments between 90 and 150 base pairs in length, Moulière and colleagues achieved a more than 2-fold median enrichment of tumor cfDNA in about 95% of cases, increasing the detection rate of clinically actionable mutations and copy number alterations in plasma cfDNA.[108]

Furthermore, ML-guided extraction of MRI features of brain tumors, characterizing their texture,[109] could complement quantitative data obtained by cfDNA improving the clinical follow-up and the rationale for therapeutic decisions.

Outstanding questions

In previous reports a negative association of baseline CSF cfDNA concentration with survival was independent of demographic or clinico-pathologic data.[15] A similar trend was reported with plasma cfDNA.[72] This raises the intriguing possibility that cfDNA release is depending on intrinsic biological features of the tumor and not just on its size. Thus, to make progress in the interpretation of liquid biopsy data a deeper insight into mechanisms of cfDNA release into the CSF or the bloodstream is desirable.

The process of nucleosome eviction might be one relevant mechanism of cfDNA increase in the periphery worth further investigation. Different genes are responsible for nucleosome eviction and consequent chromatin reshaping that increases the availability of DNA stretches for interaction with transcription factors in promoter or enhancer regions of the genome. Bromodomain protein 4 (BRD4) is one such factor, facilitating nucleosome eviction by acetylating the critical residue for nucleosome stability H3 K122.[110] Notably, BRD4, also implied in enhancing epistasis by maintaining three-dimensional chromosomal interactions,[111] is overexpressed in GBM and together with other BET bromodomain proteins is required for GBM proliferation.[112]

Another mechanism highly relevant to glioma biology is hypoxia.[113,114] Hypoxic repression of endothelial nitric-oxide synthase transcription is coupled with nucleosome eviction.[115] Nucleosome-free DNA regions (NFRs) can be established by nucleosome eviction in hypoxia-inducible promoters by hypoxia-inducible transcription factors and nucleosome re-assembly can take place hours after reoxygenation.[116] NFRs are typical configurations of chromatin in active gene promoters, and each NFR in a promoter region encompasses 100–500 base pairs, corresponding to 1–2 nucleosomes.

Final remarks

The present status of liquid biopsy in neuro-oncology is summarized in Figure 2. Analysis of CSF may provide information on differential diagnosis when surgery is not recommended or has not been informative. Plasma is more amenable to clinical follow-up, helping to decipher brain tumor modulation by tissue microenvironment and therapeutic challenges. Technical advances at three levels have the potential to improve the informative potential of plasma

cfDNA: methylation profiling, temporary BBB disruption, and increased knowledge of the biology of cfDNA shedding by the tumor.

Literature search

Data for this Review were identified by searches of PubMed, and references from relevant articles using the search terms “liquid biopsy”, “cell free DNA”, “glioma”, “brain metastases”, “liquid biopsy and CSF”, “liquid biopsy and plasma”. Abstracts and reports from meetings were not included. Only articles published in English between 1977 and March 2023 were included.

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Figure legends

Figure 1 The workflow of plasma-based and CSF-based liquid biopsy in neuro-oncology. Tumor-derived material (cfDNA, EVs, CTCs) is released in both CSF and plasma. Samples of both fluids can be obtained in the clinic by lumbar puncture or ventricular shunts (CSF) or venous blood sampling (plasma). Fresh biological fluids should be centrifuged at different speeds according to different protocols, depending on the material it is looked for, in order to separate the supernatant from the pellet and isolate the material of interest. Nucleic acids can be extracted from either EVs or CTCs and then quantified by fluorometric assay or qPCR. Once good-quality material has been obtained, a plethora of genetic analyses can be performed, from whole-genome sequencing to methylomics. Abbreviations: VS = ventricular shunt; cfDNA = cell-free DNA; EVs = extracellular vesicles; CTCs = circulating tumor cells; WBCs = white blood cells; NGS= next-generation sequencing; WES = whole exome sequencing; WGS = whole genome sequencing. Created with Biorender.com.

Figure 2 Strongpoints and caveats of plasma-based (left side of the panel) and CSF-based (right side of the panel) liquid biopsy of cfDNA. Both plasma and CSF can be precious sources of genetic material shed by brain tumors in bodily fluids. On the one hand, venous blood sampling is less invasive than CSF withdrawal, and can be easily repeated during clinical follow-up, and namely during routine outpatient visits. On the other hand, CSF generally contains larger amounts of tumor-derived genetic material and lesser amounts of genetic material released from normal cells, resulting in higher rates of mutation detection. Moreover, the total amount of cfDNA in plasma may also transiently increase due to intervening conditions (e.g., venous thromboembolism[90], inflammation[91], sepsis[91,92], focal epilepsy[94]), which should be accounted for during result interpretation. Abbreviations: cfDNA = cell-free DNA; EVs = extracellular vesicles; CTCs = circulating tumor cells; CNVs = copy number variations. Created with Biorender.com.

Table 1 A selection of relevant studies, listed in order of publication, suggesting a potential application of cfDNA-based liquid biopsies for purposes of response assessment and prognostic stratification in patients with primary and secondary CNS tumors

Reference	n	Population	Tumor Type	Biological fluid	Biomarker	Sequencing Method	Clinical application	Main Findings of the Study
De Mattos-Arruda et al.[14]	12	Adults	Primary and secondary CNS tumors	CSF	Mutations and CNVs	NGS ddPCR	Response assessment	MAF of CSF tcfDNA decreased after surgery and increased at progression
Panditharatna et al.[12]	48	Children AYA	Diffuse midline gliomas	Plasma	H3K27M mutations	ddPCR	Response assessment	MAF tracked tumor evolution and predicted relapse
Juratli et al.[18]	38	Adults	Newly-diagnosed and recurrent GBM	CSF Plasma	TERTp mutations	ddPCR	Prognosis	MAF are predictive of OS
Miller et al.[15]	85	Adults	LGG and GBM	CSF	tcfDNA detection	NGS	Prognosis	The presence of tcfDNA was associated with shorter OS
Escudero et al.[57]	13	Children Adolescents	Medulloblastoma	CSF Plasma	tcfDNA	WES ddPCR	Response assessment	CSF tcfDNA allows the study of minimal residual disease
Muralidharan et al.[22]	157	Adults	Lower grade gliomas and GBM	Plasma	TERTp mutations	ddPCR	Response assessment	MAF paralleled clinical and radiological evolution
Fontanilles et al.[73]	52	Adults	Newly diagnosed GBM or gliosarcoma	Plasma	cfDNA concentration	-	Response assessment	Median cfDNA concentrations tracked tumor evolution
Bagley et al.[72]	62	Adults	Newly diagnosed GBM	Plasma	cfDNA concentration	-	Prognosis	Preoperative cfDNA concentration correlated with PFS and OS
Liu et al.[29]	134	Children	Newly diagnosed medulloblastoma	CSF	tcfDNA detection	NGS	Response assessment Prognosis	Patients with detectable tcfDNA during treatment had worse PFS
Li et al.[78]	92	Adults	Newly diagnosed NSCLC with brain metastases	CSF Plasma	tcfDNA detection	NGS	Response assessment Prognosis	Patients with CSF tcfDNA at baseline had shorter OS
Cantor et al.[32]	28	Children	H3K27M-mutant gliomas	CSF Plasma	H3K27M mutations	ddPCR	Response assessment Prognosis	MAF variations correlate with PFS and predict targeted therapy response
Kojic et al.[19]	12	Children Preadolecents	Malignant primary brain tumors	CSF	tcfDNA	WES ddPCR	Response assessment	ctDNA correlated with disease course and clinical outcomes

Abbreviations: AYA = adolescents and young adults, cfDNA = cell-free DNA, CNVs = copy number variations, ddPCR = digital droplet PCR, GBM = glioblastoma, LGG = lower grade gliomas, MAF = mutant allelic frequencies, n = number of patients included, NGS = Next Generation Sequencing, NSCLC = non-small cell lung cancer, OS = overall survival, PFS = progression-free survival, tcfDNA = tumor-derived cell-free DNA, TERTp = TERT promoter, WES = whole exome sequencing.

Table 2 A selection of studies on liquid biopsy, listed based on order of publication, suggesting the potential use of extracellular vesicles for prognostic stratification and tumor monitoring in patients with primary and secondary CNS tumors

Reference	n	Population	Tumor Type	Biological fluid	Biomarker	Methods	Clinical application	Main Findings of the Study
Koch et al.[37]	11	Adults	GBM	Plasma	MV count	Flow cytometry, Electron microscopy	Response assessment	MV count helped to distinguish between tumor progression and pseudoprogression
Shi et al.[38]	70	n.r.	Grade 2 astrocytoma, Grade 2 ependymoma, GBM	Plasma CSF	miR-21	TEM, WB, RT-PCR	Prognosis	miR-21 levels can predict PFS and OS
Evans et al.[39]	16	Adults	GBM	Plasma	MV count	Flow cytometry, Cryoelectron microscopy	Prognosis	MV count can predict PFS and OS
Manda et al.[46]	96	Adults	HGG	Serum	EGFRvIII	BCA protein assay, TME, Flow cytometry, RT-PCR	Prognosis	EGFRvIII expression in EVs correlates with OS
Indira Chandran et al.[44]	82	Adults	HGG and LGG	Plasma	SDC1	NTA, TEM, ProSeek multiplex proximity extension assay, ELISA	Prognosis	SDC1 expression in EVs correlates with OS
Osti et al.[43]	68	Adults	GBM and other CNS tumors	Plasma	EVs count and size, proteomic profile	NTA, TEM, Mass spectrometry	Response assessment	EVs can assist in GBM diagnosis and monitoring
Zhong et al.[40]	147	Adults	HGG	Serum	miR-29b	RT-PCR	Prognosis	miR-29b levels correlate with OS
Batool et al.[35]	40	Adults	HGG	Plasma	EGFRvIII	ddPCR	Response assessment	EGFRvIII mutant copies assisted in response assessment
Khristov et al.[41]	82	Adults	GBM	Plasma	IL13Ra2	ELISA	Prognosis	IL13Ra2 levels in plasma correlate with OS
Dobra et al.[42]	222	Adults	GBM, Meningiomas, Brain metastases	Serum	MMP-9	NTA, TEM, WB, LC-MS, ELISA	Prognosis	MMP-9 levels correlate with OS

Abbreviations: cfDNA = cell-free DNA, ddPCR = digital droplet PCR, ELISA = enzyme-linked immunosorbent assay, EVs = extracellular vesicles, GBM = glioblastoma, HGG = high-grade gliomas, LC-MS = liquid chromatography–mass spectrometry, LGG = low grade gliomas, MV = microvesicles, n = number of patients included, n.r. = information not reported, NTA = nanoparticle tracking analysis, OS = overall survival, PFS = progression-free survival, RT-PCR = Reverse Transcription PCR, TEM = transmission electron microscopy, WB = Western Blot.

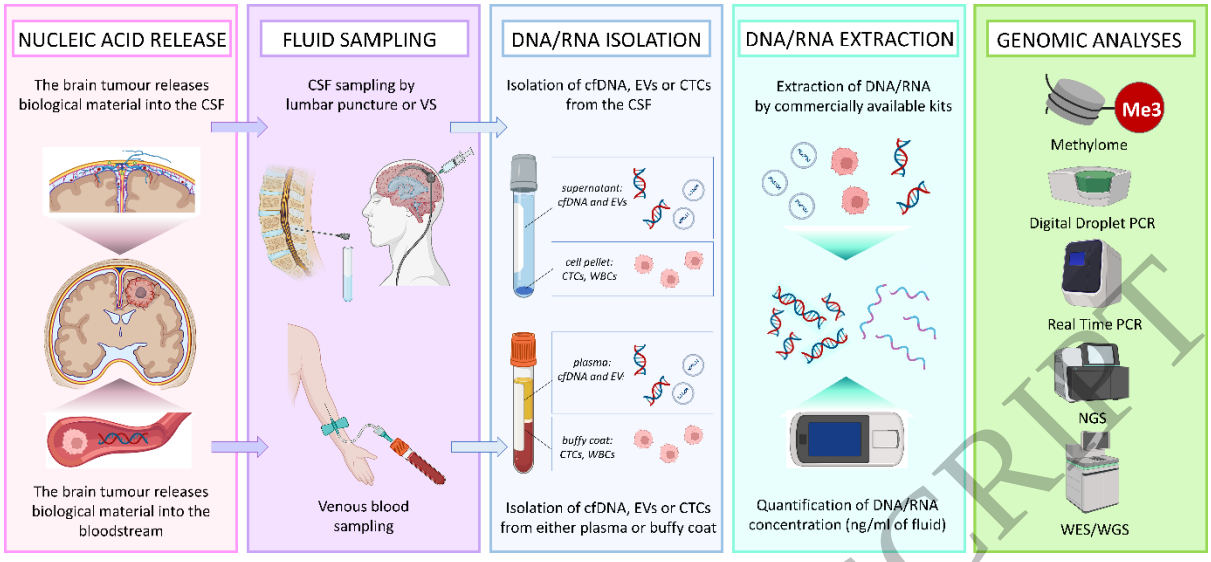

Figure 1
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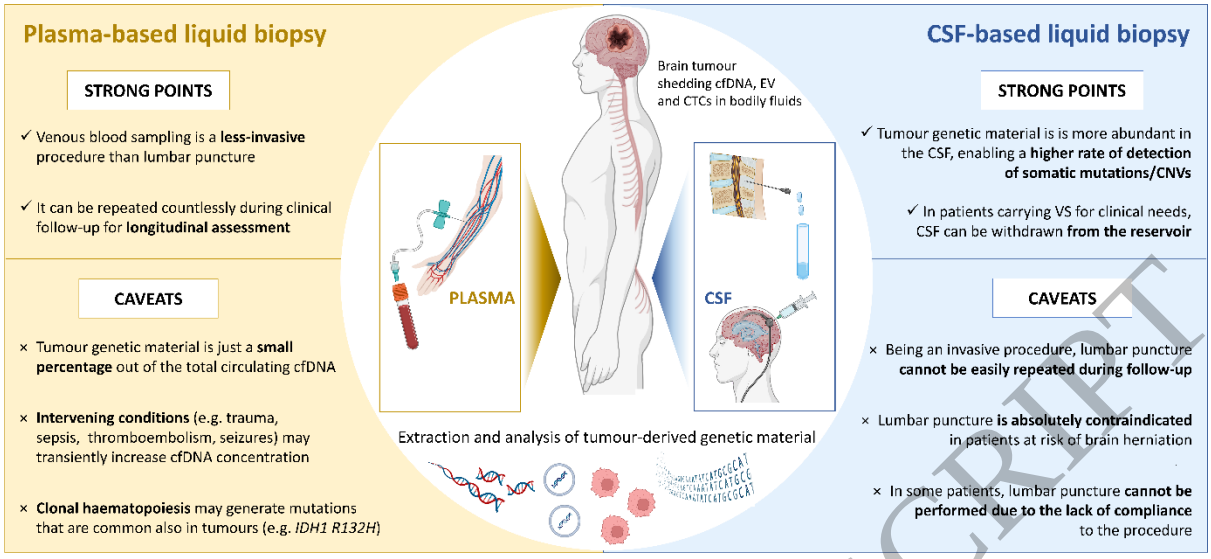

Figure 2
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