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The Quest for Non-Invasive Diagnosis: A Review of Liquid Biopsy in Glioblastoma

Maria George Elias [1,2,3]**, Harry Hadjiyiannis** [1,3]**, Fatemeh Vafaee** [4,5]**, Kieran F. Scott** [3]**, Paul de Souza** [1,3,6]**,Therese M. Becker** [3,7,8] **and Shadma Fatima** [1,3,4,]*****

[1] Ingham Institute for Applied Medical Research, Liverpool, NSW 2170, Australia

[2] School of Science, Western Sydney University, Locked Bag 1797, Penrith South DC, Sydney, NSW 2751, Australia

[3] School of Medicine, Western Sydney University, Locked Bag 1797, Penrith South DC, Sydney, NSW 2751, Australia

[4] School of Biotechnology and Biomolecular Science, University of New South Wales, Sydney, NSW 2052, Australia

[5] University of New South Wales, Deputy Director (Science), UNSW AI Institute, UNSW, Sydney, NSW 2052, Australia

[6] Nepean Clinical School, The University of Sydney, Sydney, NSW 2747, Australia

[7] Centre for Circulating Tumour Cell Diagnostics and Research, Ingham Institute for Applied Medical Research, Liverpool, NSW 2170, Australia

[8] South Western Sydney Clinical School, University of New South Wales, Liverpool, NSW 2170, Australia

***** Correspondence: shadma.fatima@inghaminstitute.org.au; s.fatima@unsw.edu.au; Tel.: +61-410063786

Abstract

Glioblastoma multiforme (GBM) remains the most common and fatal primary brain tumour, with low survival rates due to its heterogeneous nature. Traditional diagnostic methods, such as magnetic resonance imaging (MRI), computed tomography (CT) scans, and invasive tissue biopsies for histopathological analysis, are often insufficient in differentiating treatment-related changes from tumour progression, leading to misdiagnosis and delays. Repeated biopsies are impractical for ongoing monitoring due to their invasive nature. A promising alternative is liquid biopsy, a minimally invasive procedure that analyses biofluids like blood and cerebrospinal fluid (CSF) for tumour-related biomarkers. Tumours release a variety of components into the bloodstream, including circulating tumour cells (CTCs), circulating tumour DNA (ctDNA), microRNAs (miRNAs), extracellular vesicles (EVs), and proteins, which can cross the blood-brain barrier and provide a non-invasive means of assessing genetic, epigenetic, proteomic, and metabolomic markers associated with GBM. Liquid biopsy can be performed multiple times throughout the disease course, offering a rapid way to gather critical and changing tumour information. However, challenges remain in the clinical implementation of liquid biopsies for GBM diagnosis, including low levels of circulating biomarkers and the lack of standardised assays for biofluid collection and analysis. Combining multiple biomarkers from various bio-elements, such as CTCs, ctDNA, miRNAs, and EVs, could enhance sensitivity and specificity, addressing some of these limitations. The integration of artificial intelligence (AI) for analysing liquid biopsy biomarkers holds great promise in overcoming these challenges. AI can enhance biomarker identification, improve diagnostic accuracy, and expand the clinical utility of liquid biopsy in GBM management. Further research, and large-scale clinical validation are needed to optimise these approaches. This review explores the potential of GBM biomarkers found in blood and CSF samples, focusing on their applications in diagnosis, prediction, and prognosis, and highlights recent advancements, challenges, and future perspectives, including the integration of AI to improve outcomes in GBM management. Liquid biopsies bridge the gap between invasive methods and emerging technologies, offering transformative potential for GBM diagnosis and treatment.

1. Introduction

Glioblastoma Multiforme (GBM) is the most common and aggressive primary brain tumour, with a median survival of around 15 months despite intensive treatments including surgery, radiation, and chemotherapy [1]. Its highly invasive nature, resistance to therapy, and recurrence make it one of the most challenging cancers to treat. GBM is also marked by substantial molecular heterogeneity, including gene mutations such as IDH-R132H, H3-K27M, and EGFR (EGFRvIII) amplification complicating treatment approaches and prognosis. Moreover, the blood-brain barrier (BBB) further limits the effectiveness of conventional therapies and monitoring methods [2–4].

Diagnosing GBM typically includes neuroimaging, followed by histopathological and molecular analysis for confirmation[5–7]. However, both imaging and tissue-based methods have limitations, such as distinguishing between relapse and pseudo progression, which are lesions related to treatment simulating recurrence of GBM; a continuous challenge [8]. These challenges underscore the need for alternative, non-invasive diagnostic tools, such as liquid biopsy, which has surfaced as a promising approach to detect early recurrence for GBM patients, principally through blood tests, urine, or cerebrospinal fluid (CSF), providing real-time insights into tumour dynamics and treatment response[9,10]. Liquid biopsies comprise the detection and quantification of tumour related content in biofluids that is released by tumours, such as circulating tumour cells (CTCs), circulating tumour DNA (ctDNA), circulating tumour microRNA (miRNA), extracellular vesicles (EVs) and nucleosomes or in response to tumours, all of which can provide critical insights into GBM progression and recurrence [11].

Being minimally or non-invasive, liquid biopsy offers a valuable alternative to invasive tissue biopsies for detecting early recurrence, monitoring minimal residual disease (MRD), and guiding treatment strategies in GBM. While the BBB hinders the amount of tumour-derived entities that may be found in body fluids, ongoing research aims to increase sensitivity and specificity of liquid biopsy tests, making them a promising tool for advancing clinical management of GBM. This review will explore recent advancements in liquid biopsy technologies and their potential for enhancing the diagnosis and treatment of GBM.

2. Circulating Biomarkers of Glioblastoma from Liquid Biopsies

2.1. Circulating Tumour Cells

CTCs are individual cells or cell clusters that are released from primary or metastatic solid tumours into bodily fluids. When disseminated into the bloodstream and other body fluids, they may contribute to metastasis and can be used as biomarkers for cancer detection, prognosis, and therapeutic monitoring[9,12]. The analysis of CTCs emerged as a potential tool for monitoring MRD in cancer patients[13–15]. Higher levels of CTCs are directly related to severity and spread of multiple cancers[16]. Key methods for CTC enumeration, which quantify CTC numbers include sensitive assays like CellSearch® (Menarini Silicon Biosystems, Italy), which detects as few as one CTC per 10 mL of blood. However, **CellSearch**® uses antibody-mediated capture, mainly targeting epithelial cell adhesion molecule **(EpCAM)** on CTC membrane surfaces using a positive selection method[17–21].

EpCAM is not expressed in GBM tumours due to their predominantly mesenchymal phenotype[12,22]. Hence, alternative techniques for isolation of CTCs in GBM patients have been explored (Table 1). One such method is negative selection, which enriches for CTCs by eliminating leukocytes from whole blood by means of antibodies against leukocyte antigens[9,19,29,32]. Several different techniques of CTC isolation have been used which are dependent on the CTCs physical properties[23,24]. These include density-gradient centrifugation[27,28], spiral microfluidic technology[30,31] and chip technology[21]. Although promising, it is important to highlight how rare

CTC detection in the peripheral circulation of GBM patients actually is. Reported detection rates vary widely, with some studies identifying CTCs in as few as 20-40% of GBM patients, depending on the sensitivity of the detection method and sample volume and timing (20.6% - 77% of patients with detectable CTCs, Table 1). Along with the complexity of isolating CTCs from peripheral blood, this rarity underscores the need for highly sensitive and specific detection technologies to validate their clinical utility as reliable biomarkers. It also further complicates standardisation efforts and underlines the need for optimisation and harmonisation of isolation protocols.

CTC analysis methods for GBM, other than enumeration, include, molecular profiling (e.g., next generation sequencing (NGS), Reverse Transcriptase-PCR (RT-PCR)), immunophenotyping using GBM-specific markers (such as GFAP, Nestin, Sox2, EGFR, GLAST)[21,27,29–31], single-cell sequencing[27], and epigenetic profiling[25], with CSF-based analysis often offering higher sensitivity than blood[26–28]. These methods enable the detection of clinically-relevant alterations, such as IDH1 mutations and MGMT promoter hypermethylation, which are important for prognosis and for guiding targeted therapy decisions in GBM[19].

Monitoring CTC kinetics over time can provide insights into the effectiveness of treatment and the risk of disease recurrence. Changes in CTC count, phenotype or genetic profile can act as early indicators of therapeutic response of tumour progression[21,27–29,31,32] (Table 1).

Table 1. CTC isolation and analysis in patients with GBM.

Isolation method	Patient numbers	Markers used to verify GBM origin	Findings	References and publication date
CTC-iChip microfluidic technology. Leukocyte depletion using magnetically-tagged anti-CD45 and anti-CD16 antibodies.	33	SOX-2, EGFR, c-MET, A2B5 tubulin β-3	Isolated CTCs from peripheral blood of 39% (13 of 33) GBM patients. Greater CTC counts in patients with progressive disease relative to stable disease. CTCs have a mesenchymal phenotype.	[22] (2014)
Density gradient centrifugation	141	Single-cell genomics for common GBM mutations, GFAP staining, tumour specific anomalies like amplification of EGFR gene and gains and losses in chromosomes 7 and 10 genomic regions by chromosomal and array CGH on whole genome amplification.	Isolated CTCs from 20.6% (29/141) of patients. A convenient diagnostic tool for identifying patients with extracranial tumour cell spread and indicates that CTCs could be used to monitor the progression of glioblastoma.	[29] (2014)
Density gradient centrifugation using the OncoQuick® system.	11	Telomerase-based test was used to identify CTCs.	CTCs detected in 72% (8 of 11) of patients before radiotherapy but dropped to 8% (1 of 8) in post-radiotherapy patients. This suggests that CTCs may be useful to monitor the progression of cancers before and after therapies.	[30] (2014)
Immunoaffinity-based methods. CTC separation with a matrix and negative depletion of white blood cells using immunomagnetic beads.	31	Polyploidy chromosome-8-positive detection was employed as a positive measure for CTCs using subtraction enrichment and *immunostaining-fluorescence in situ hybridization (SE-iFISH)*, in addition to GFAP-positive or GFAP-negative cells and CD45-negative cells grading to confirm cell origin.	CTCs were detected in 77% (24 of 31) of GBM patients. Monitoring treatment using CTCs was slightly better than MRI in distinguishing radionecrosis from recurrence of glioma. CTCs can dynamically monitor the microenvironment of gliomas which is a significant complement to radiographic imaging.	[31] (2016)

Immunotargeted enrichment of MSP & MCAM expressing cells.	13	CTCs were isolated based on cell surface MSP and MCAM and identified by probing for GLAST and/or GFAP expression.	≥ 1 CTCs were detected in 69% of patients (9/13), using the combination of 2 isolation and 2 identification markers increased CTC detection.	[21] (2020)
Immunopheno-typing. CTCs were isolated by size separation using a Parsortix® microfluidic cassette.	13	CTCs were isolated based on no expression of CD45, while expressing EGFR, Ki67, and EB1 microtubule associated protein. For confirmation, the GBM CTC clusters and a biopsy from the primary tumour of the patient were stained with GBM marker SOX-2.	CTC clusters identified in 53.8% of 13 GBM patients. GBM markers validated that multicellular CTC clusters can be formed and pass the BBB in patients with GBM to reach peripheral circulation and be used for monitoring.	[32] (2018)
Spiral microfluidic technology	20	CTCs exhibited characteristic molecular features of GBM, such as EGFR amplification and mutations in TP53 and IDH1.	The study found that CTCs could be isolated from both early- and late-stage GBM patients, highlighting the potential of this technique for non-invasive monitoring of tumour progression.	[33] (2021)
CTC Subtraction enrichment/depletion with magnetic immunoaffinity beads and immunostaining-FISH	22	Detection of CTCs to differentiate between treatment-induced necrosis and tumour recurrence in brain gliomas. CTC detection outperformed both DSC-MRP and MET-PET in diagnostic accuracy. Additionally, it showed potential for predicting recurrence in one patient.	The mean CTC count was significantly higher in patients with tumour recurrence (6.10 ± 3.28) compared to those with treatment necrosis (1.08 ± 2.54). A threshold CTC count of 2 provided 100% sensitivity and 91.2% specificity (AUC = 0.933) for identifying tumour recurrence. CTC detection could be a valuable tool for distinguishing tumour recurrence from necrosis, warranting further validation in larger clinical studies.	[34] (2021)

These studies demonstrate the potential of CTCs as a tool for cancer diagnosis and prognosis, however, there are still challenges that need to be addressed: (i) Low abundance: CTCs are present in very low numbers in the bloodstream being 1 CTC in 10^9 normal blood cells per 10 mL of blood, making their isolation and analysis challenging[9,18]. Low abundance can lead to false-negative results and reduce the sensitivity of CTC-based assays. (ii) Heterogeneity: CTCs show intra- and inter-patient heterogeneity and may differ in morphology, phenotype, and genetic profile. This heterogeneity complicates the identification and characterisation of CTCs, and it may require the use of multiple markers or assays to detect different subsets of CTCs[35,36]. (iii) Technical variability: The isolation and analysis of CTCs require specialised equipment and protocols, and it can affect the accuracy and reproducibility of CTC-based assays. The choice of isolation and analysis method, as well as the handling and storage of blood samples, can all impact the results of CTC assays[37,38]. (iv) Lack of standardisation: There is currently no standardised protocol for the isolation and analysis of CTCs, and different studies may use different methods or markers to detect CTCs. This lack of standardisation can make it difficult to compare results across studies and to establish the clinical utility of CTC-based assays[39]. (v) Cost and accessibility: CTC-based assays can be costly and require specialised equipment and expertise, which may limit their availability and accessibility in a clinical setting[40].

Overcoming these barriers will require continued innovation in sensitive and specific CTC isolation and analysis techniques, along with greater collaboration between researchers and clinicians in establishing the clinical utility of this approach. By integrating CTC-based assays with other clinical and pathological parameters, and rigorously validating these methods in clinical trials, the clinical utility and potential for earlier accurate cancer detection and monitoring tumour progression may be determined.

2.2. Circulating Tumour DNA

Cell-free DNA (cfDNA) are short DNA fragments of 140-170 base pairs (bp) present in body fluids. In cancer patients, a portion of cfDNA originates from tumour cells following apoptosis or necrosis, referred to as **circulating tumour DNA (ctDNA)**[9,10,18,41,42]. Representing a promising tool for non-invasive cancer diagnosis, particularly in GBM, ctDNA consists of shorter DNA fragments (< 145 bp) that carry the genetic and epigenetic signatures of the originating tumour[12,18]. A number of studies have validated that ctDNA reflects the genetic profile of the original tumour, showing uniformity with its mutational profile and that of the corresponding tumour tissue across various cancers, including GBM[9,43–45], thereby offering insights into tumour genetics and treatment response (see Table 2). Key detection methods include assessing ctDNA levels and known point mutations using polymerase chain reaction (PCR)-based method such as digital droplet PCR (ddPCR), and next-generation sequencing (NGS) or whole genome sequencing (WGS), method that detects novel or unknown mutations[46–48]. ddPCR is particularly sensitive for identifying low-frequency mutations such as IDH1, TERT promoter mutations, and EGFRvIII in plasma samples[48]. NGS enables comprehensive genomic profiling, detecting a broad spectrum of mutations and copy number alterations[49]. Methylation-specific PCR targets epigenetic modifications, like MGMT promoter methylation, which are relevant for GBM prognosis and therapy selection[47].

Detecting ctDNA in GBM is challenging due to tumour's location and the BBB, which decreases yield of ctDNA in peripheral circulation. Despite these obstacles, significant work has been done towards ctDNA detection by employing highly sensitive molecular technologies as described in Table 2. It is noteworthy to mention the baseline level of cfDNA is greater in serum compared to plasma, which may be in result of contamination with DNA that is released from lysed immune cells, thus plasma samples are preferred for studying ctDNA[46–48]. While plasma ctDNA alone may not yet be sufficiently sensitive for reliable GBM diagnosis or monitoring, detectable levels offer promise for non-invasive molecular profiling and could help identify actionable mutations and resistance mechanisms, potentially improving treatment strategies. Studies have shown that ctDNA can be detected in a subset of GBM patients, though plasma detection rates are generally lower than in other cancers, likely due to the BBB limiting ctDNA release into the bloodstream[50]. Piccioni et al. found ctDNA mutations in the blood of 50% of brain tumour patients, with 55% of these being GBM cases[51]. However, dependent on patient cohort and cfDNA extraction method 75.0% sensitivity (95% CI: 64.1%–84.0%) and 88.7% specificity (95% CI: 77.0%–95.7%) has been achieved for detection of the IDH1 mutation R132H, and negativity for this mutation is a key classifier of GBM[52,53].

EGFRvIII ctDNA was detectable in plasma and can serve as a dynamic biomarker for preoperative and postoperative assessment, as well as for monitoring disease recurrence in GBM patients[54]. Zill et al. used next-generation sequencing of plasma ctDNA in 107 GBM patients to identify actionable mutations, supporting the use of ctDNA sequencing for personalised cancer treatment[55]. Liu et al., and Miller et al., further demonstrated that WGS of CSF ctDNA achieves high detection rates and can identify clinically relevant mutations, reinforcing its utility as a minimally invasive biomarker for GBM monitoring[56,57].

Furthermore, studies have demonstrated that CSF is a more reliable source for ctDNA in GBM patients compared to plasma, overcoming the limitations imposed by the BBB in regard to ctDNA release into the bloodstream[58–60]. For instance, ctDNA was detected in 61% of CSF samples compared to 37% in serum of 19 GBM patients using methylation-specific PCR[61], while nested PCR allowed for detection in 92% of CSF samples compared to only 8% in plasma samples of 38 GBM patients [62]. Another study using WGS on CSF samples of 11 GBM patients demonstrated a 100% ctDNA detection rate[63]. These findings highlight the superior sensitivity of CSF-based liquid biopsies, likely due to the proximity of CSF to the brain tumour and the reduced interference from background cfDNA found in blood[9,12,18,64]. However, CSF collection via lumbar puncture is invasive and may not be suitable for all patients[18]. As alternatives, less invasive fluids like nasal secretions or saliva may provide an alternative to CSF, offering a less invasive means to access brain-derived biomarkers but needed further investigation.

Urine has also emerged as a non-invasive source of ctDNA for GBM diagnosis, offering the advantage of easy and repeatable collection throughout treatment[10]. Following renal filtration, ctDNA can be detected as transrenal DNA (trDNA), enabling molecular diagnostics through urinary DNA analysis[10,65]. Several urinary biomarkers—including matrix metalloproteinases (MMP-2, MMP-9), neutrophil gelatinase-associated lipocalin (NGAL), and vascular endothelial growth factor (VEGF) have demonstrated high sensitivity (95.2%), specificity (95.7%), and accuracy (92.5%) in detecting primary brain tumours, including GBM (see Table 2). Notably, elevated levels of these urinary markers in GBM patients have been shown to correlate with their expression in tumour tissue and, in CSF (for MMP-9 expression)[65–68]. In addition, urine-based detection of ctDNA epigenetic alterations, such as **DNA methylation**, offers further potential. Methylation-specific PCR enables the quantification of tumour-specific methylation, such as **O6-methylguanine-DNA methyltransferase (MGMT) promoter methylation**[69,70], a well-established predictor of GBM patient response to alkylating agents like temozolomide (TMZ)[71–73].

In conclusion, ctDNA analysis has shown significant promise as a tool for detecting and monitoring genetic alterations in brain cancers, although some challenges still need to be addressed. These include variability in ctDNA levels depending on the stage and anatomical location of cancer, as well as its rapid degradation in circulation and sensitivity to pre-analytical factors, which can complicate collection and analysis. Overall, detection of ctDNA in CSF and plasma provides valuable insights into tumour evolution, resistance mechanisms, and potential therapeutic targets, offering a non-invasive approach to guide personalised treatment for CNS cancers. Further advancements in ctDNA isolation, sequencing methods, and clinical validation will be crucial to unlock its full potential for early diagnosis, treatment monitoring, and personalised cancer management.

Table 2. ctDNA isolation and analysis in patients with GBM.

Isolation method	Patient numbers	Markers used to verify GBM origin	Findings	References and publication date
Guardant360® and digital NGS	171	NGS targeting 54 cancer related genes, including assessments for copy number variants in EGFR, ERBB2, and MET.	Of the 33 patients diagnosed with GBM, 73% had unaltered ctDNA, 24% had one alteration and 3% had two or more alterations.	[50] (2016)
Guardant360® and digital NGS	222	Single nucleotide variants were detected in 61 genes, with amplifications detected in *ERBB2, MET, EGFR*.	ctDNA mutations were detected in blood samples from 55% of GBM patients.	[51] (2019)
Illustra triplePrep Kit (GE healthcare BioSciences Corp) and WGS	13	EGFRvIII mutation characterised by a deletion on exons 2 through 7.	EGFRvIII mutant DNA detected in the plasma of GBM patients, with its presence correlating with the mutation in tumour tissue.	[54] (2013)
DNA extraction and NGS	107	Tumour-specific mutations such as EGFRvIII	ctDNA detection rate was 51% Genomic alteration in the ctDNA of patients highlight the potential of guiding personalised cancer treatment.	[55] (2018)
DNA extraction and methylation specific PCR assay	19	MGMT promoter methylation GBM patients serum and cerebrospinal fluid CSF samples for ctDNA detection using methylation specific PCR assay,	Detected 37% ctDNA in serum and 61% ctDNA in CSF. MGMT promoter methylation was detected with higher sensitivity in CSF (72.0%) compared to serum (41.7%), suggesting CSF as a promising tool for early diagnosis, treatment monitoring, and recurrence detection.	[61] (2015)
DNA extraction and nested PCR-based assays	38	ctDNA was analysed for TERT promoter mutations (C228T and C250T) and IDH hotspot mutations.	Detected 8% (3 of 38 patients) ctDNA in plasma and 92% (35 of 38 patients) ctDNA in CSF.	[62] (2018)

DNA extraction and WGS	11	Tumour-specific mutations in the ctDNA extracted from CSF.	100% ctDNA detection rate in CSF.	[63] (2015)
DNA extraction and WGS	13	Copy number alterations in ctDNA.	50% ctDNA detection rate in CSF. Identified copy number alterations in the ctDNA, which closely reflected tumour genetic profile. Fragmentation patterns in CSF-derived ctDNA Copy number alterations in ctDNA were consistent with tumour tissue.	[74] (2018)
DNA extraction and NGS	16	IDH1/2 and 1p/19q codeletion.	ctDNA detected in 49.4% of CSF samples. Genetic alterations detected closely matched those found in tumour biopsies.	[75] (2018)
DNA extraction and NGS	NA	IDH1 (R132H variant), TERT promoter (C228T mutation), TP53, ATRX, H3F3A and HIST1H3B.	CSF ctDNA more accurately reflected BM mutations, detecting all mutations in 83.33% of cases versus 27.78% for plasma ctDNA. CSF ctDNA more accurately reflected BM mutations, detecting all mutations in 83.33% of cases versus 27.78% for plasma ctDNA. Mutant allele frequency (MAF) in CSF ctDNA strongly correlated with BM tumour size (r = 0.95) and was higher than in plasma ctDNA (38.05% vs. 4.57%). MAF and tumour mutational burden in CSF ctDNA closely matched BM values (r = 0.96 and 0.97, respectively). CSF ctDNA exhibited superior concordance with BM (99.33%) compared to plasma ctDNA (67.44%), improving the identification of clinically relevant mutations. However, for multiple BM, plasma ctDNA performed well, achieving a 93.01% concordance, comparable to CSF ctDNA.	[76] (2023)
DNA extraction and MSK-IMPACT™, a NGS assay	711	The distribution of clinically actionable somatic alterations was consistent with tumour-type specific alterations across the AACR GENIE cohort.	Genetic alterations were detected in 53% (489/922) of CSF samples from patients with confirmed CNS tumours, while none of the 85 samples from patients without CNS tumours contained detectable ctDNA. The identified mutations aligned with tumour-type-specific alterations observed in the AACR GENIE cohort. Repeated ctDNA testing revealed clonal evolution and resistance mechanisms, and the presence of ctDNA linked to reduced overall survival after CSF collection.	[77] (2024)
QIAamp Circulating Nucleic Acids kit (QIAGEN), Western blot,	64	Patient-derived orthotopically implanted xenograft models of GBM.	Analysis of fragment length profiles of host (rat) and tumour (human) ctDNA identified a peak at 145 bp in the human DNA fragments, indicating a	[65] (2019)

histopathology, and immunochemistry, digital PCR, and shallow whole genome sequencing were utilised			difference in the origin or processing of the ctDNA. The concentration of ctDNA correlated with cell death only after treatment with TMZ and radiotherapy. ddPCR detection of plasma tumour mitochondrial DNA (tmtDNA), an alternative to detection of nuclear ctDNA, improved plasma DNA detection rate (82% versus 24%) and allowed detection in CSF and urine. The total amount of tmtDNA in the plasma (558 copies) is ~13 times higher than in the CSF (43 copies), showing therefore that the BBB does not prevent significant amounts of tumour DNA	
QIAamp DNA micro kit (Qiagen) and amplicon sequencing	20	DH1, IDH2, TP53, TERT, ATRX, H3F3A, and HIST1H3B gene mutations	The genomic analysis in CSF extracted ctDNA facilitates the diagnosis of diffuse gliomas into subtypes to support the surgical and clinical management of these patients.	[75] (2018)
QIAamp Circulating Nucleic Acid kit and NGS	26	Cancer genomic panel sequencing on the CSF-derived ctDNA.	A high detection rate of ctDNA (24/26, 92.3%) was observed in CSF. ctDNA mutations had high concordance rates with tumour DNA, especially in non-copy number variations and in GBM. CSF ctDNA TMB also exhibited a strong correlation with tumour DNA TMB ($R^2 = 0.879$, $P < 0.001$), particularly in GBM ($R^2 = 0.992$, $P < 0.001$).	[78] (2022)
DNeasy Blood and Tissue Kit (Qiagen), and targeted DNA sequencing by Oxford Nanopore Technology MinION device.	12 paediatric high-grade glioma patients and 6 controls	Analysis of ctDNA with a handheld platform (Oxford Nanopore MinION) to quantify patient-specific CSF ctDNA variant allele fraction (VAF)	Nanopore demonstrated 85% sensitivity and 100% specificity in CSF samples (n = 127 replicates) with 0.1 femtomole DNA limit of detection and 12 h results, all of which compared favourably with NGS. Multiplexed analysis provided concurrent analysis of H3.3A (H3F3A) and H3C2 (HIST1H3B) mutations in a non-biopsied patient and results were confirmed by ddPCR. Serial CSF ctDNA sequencing by Nanopore demonstrated correlation of radiological response on a clinical trial, with one patient showing dramatic multi-gene molecular response that predicted long-term clinical response.	[79] (2020)

2.3. Nucleosomes

Chromatin structure was first described in 1974 as repeating nucleosome units. Each nucleosome consists of an octamer of two copies, each of the core histones H2A, H2B, H3 and H4, wrapped by 146-147 base pairs of DNA[80,81]. Nucleosomes are generated by DNA associated with proteins, including histone H1. Histones have a globular domain for histone-histone interactions and flexible, positively charged tails (20-35 amino acids) that undergo post-translational modifications (PTMs)[82]. As the basic units of chromatin, nucleosomes regulate key nuclear processes like transcription, replication and repair, primarily through PTMs[83]. These modifications influence

gene expression on an additional level to DNA methylation, and activating oncogenes or silencing tumour suppressor genes may contribute to cancer development and progression[83,84]. Histone PTMs include methylation, acetylation, phosphorylation, ubiquitination, sumoylation, glycosylation, homocysteinylation, and crotonylation[85]. Methylation may activate or repress gene expression depending on the site and degree[83], while acetylation generally promotes transcription by neutralising lysine's positive charge, reducing chromatin compaction and enhancing accessibility for transcription factors[85]. The interplay of these PTMs forms a "histone code" read by cellular proteins to control transcription, replication, and repair, with core histone variants adding further complexity[85,86].

Nucleosomes and their PTMs can be detected in plasma or serum using immunoassays like chemiluminescence immunoassay (ChLIA) or enzyme-linked immunosorbent assay (ELISA) (Table 3). These assays detect specific biomarkers from complex matrices, and can be applied on automated platforms which would allow faster and more reproducible results[87]. Cancer cell death, including that of GBM cells results in the release of nucleosomes into the bloodstream, which are transported as mono- or oligo- nucleosomes bound to ctDNA.

Cancer patients have a greater count of circulating nucleosomes due to the increase in cellular turnover in comparison to healthy controls and due to the cytotoxic effect of treatments which lead to cell death[83]. The high levels of nucleosomes are not explicit to cancer or GBM per se, since it is also linked to trauma, stroke and sepsis which limits its clinical use as a unique biomarker for GBM detection. The latest studies have however, reported that the PTMs found on circulating nucleosomes may have cancer specificity and are thus be investigated as biomarkers[88].

Enzymes that regulate PTMs are often dysregulated in GBM. Histone deacetylases and demethylases like lysine-specific histone demethylase 1, can alter the epigenetic status of brain cells, influencing cancer development and progression [88,89]. In paediatric high-grade gliomas, recurrent histone mutations are common. The H3K27M mutation, a lysine-to-methionine substitution at position 27 of H3.1 or H3.3 histone genes, is associated with childhood diffuse intrinsic pontine glioma, disrupting polycomb repressive complex 2 function, leading to global H3K27 methylation loss and altered gene expression[90]. H3G34R/V mutations, involving glycine 34 substitutions in H3.3 are seen in gliomas of the cerebral hemispheres in both children and adults causing redistribution of the activating H3K36 methylation mark and transcriptional dysregulation[88,90].

Another epigenetic marker dysregulated in GBM is histone 3 lysine 4 (H3K4), which is decreased in severe GBM cases, leading to gene repression[89]. Acetylation of histone 3 at lysine 18 (H3K18Ac), is another common PTMs with dysregulated expression seen in a number of cancers including GBM, where lower levels identify better prognosis for the patient[91]. Accordingly, a profile of PTMs of circulating nucleosome linked histone modifications has potential to be studied in GBM patients which would be in addition to MRI, a guide to diagnosing and monitoring tumour progression in GBM patients[9].

Nucleosomes are generally less useful for cancer diagnosis due to elevated levels in benign diseases. However, in gastrointestinal cancers, nucleosome levels correlate with tumour stage and metastasis. Nucleosomes are more valuable for therapy monitoring, as their levels decrease in remission and increase with disease progression during chemotherapy and radiotherapy, a pattern observed in lung, pancreatic, colorectal cancers, and haematological malignancies [92,93].In GBM, circulating nucleosomes in serum and CSF have shown potential for diagnosis and prognosis. Patients developing cerebral oedema post-surgery exhibited a ~200-fold rise in CSF nucleosomes. Monitoring these levels may help detect complications and track tumour progression and treatment response[94]. However, further research is needed to refine their specificity and clinical application, especially in distinguishing cancer from other conditions.

Collectively, circulating nucleosomes and their PTMs present a promising avenue for GBM detection and monitoring. While total nucleosome levels in plasma and CSF lack disease specificity, unique histone modifications, such as H3K27M and H3K18Ac, provide greater diagnostic potential. Emerging technologies, including immunoassays and single-molecule detection methods, have

enabled precise profiling of nucleosome-bound epigenetic markers, distinguishing GBM patients from healthy individuals. These findings suggest that nucleosome-based liquid biopsies could serve as a valuable adjunct to MRI and conventional diagnostics, particularly in monitoring disease status and therapy response. Additionally, nucleosome levels may help identify postoperative complications, such as cerebral oedema, offering further insight into tumour progression and treatment outcomes. However, further validation in larger clinical studies is necessary to refine their specificity and clinical application, especially in differentiating cancer from other conditions.

Table 3. Nucleosome isolation and analysis in patients with GBM.

Isolation method	Patient numbers	Markers used to verify GBM origin	Findings	References
Cell Death Detection ELISA Plus kit	10	NA	Pre-therapeutic nucleosome levels in both serum and CSF did not significantly differ among GBM patients and control groups. **Postoperative Increase**: In GBM patients, nucleosome levels in serum and CSF increased moderately during the week following surgery and intracavitary chemotherapy. **Cerebral Oedema Correlation**: Three out of ten GBM patients developed cerebral oedema post-surgery. In these patients, CSF nucleosome levels increased almost 200-fold, peaking on day 3 postoperatively. In contrast, the seven patients without oedema exhibited only slight increases in nucleosome levels. **Clinical Implication**: Monitoring CSF nucleosome levels may serve as an indicator for postoperative complications such as cerebral oedema in GBM patients.	[94]
Single-molecule technology to detect and monitor plasma-circulating nucleosomes	NA	H3-K27M mutation and mutant p53	The single-molecule analysis revealed epigenetic patterns unique to diffuse midline glioma, enabling differentiation from healthy individuals and patients with other cancer types. This approach profiles multiple histone modifications on individual nucleosomes from less than 1 mL of plasma, revealing epigenetic patterns unique to glioma that significantly differentiate these patients from healthy individuals and those with other cancer types. The detection strategy demonstrated a correlation with MRI measurements and ddPCR assessments of ctDNA, highlighting its potential utility in non-invasive treatment monitoring. Suitable for paediatric patients and scenarios where sample volume is limited.	[95]

2.4. Circulating Tumour microRNA

Circulating tumour microRNA (miRNA), is the most common small non-coding RNA, typically 21-23 nucleotides long, and regulates up to 30% of protein-coding genes[96,97]. It plays a key role in cancer by downregulating tumour suppressor genes. miRNAs are highly stable and detectable in bodily fluids such as blood, urine, and CSF, and are also present in extracellular vesicles (EVs)[12,98–100].

Altered expression of specific miRNAs have been found to distinguish between GBM patients and lower-grade glioma patients (Table 4). For instance, downregulation of miR-125b, miR-16, miR-497, miR-128, miR-342-3p, miR-205 and upregulation of miR-210, miR-182, miR-20a-5p, miR-454-3p, miR-106a-5p, miR-181b-5p have been linked to GBM pathophysiology[101–108]. Thus, miRNA dysregulation in GBM involves both upregulation of oncogenic miRNAs ("oncomirs") and downregulation of tumour-suppressive miRNAs, each playing distinct roles in tumour biology.

Most miRNAs which show an increase in their expression level in GBM are oncogenic and are known as "oncomirs"[109]. Oncomirs usually promote tumour growth by inhibiting tumour suppressor genes and genes linked to cell differentiation or apoptosis. The first oncomir which showed significantly increased levels in GBM tissue samples compared to normal tissue was miR21. It can target several tumour suppressors such as PTEN[110]. The levels of miR-21 in GBM tissue and plasma samples was higher in grade II and III GBM samples compared to controls, yet there was no significant change between pre-surgery and post-surgery sample measures, while miR21 level was decreased after chemo irradiation therapy [101].

Several miRNAs function as tumour suppressors and are commonly downregulated in GBM patients[111]. Tumour-suppressive miRNA such as Let-7, which targets oncogenes like K-RAS and MYC inhibit GBM cell proliferation by interfering with histone methyltransferase EZH2[112–114]. miR-128 and miR-342-3p, significantly reduced in the plasma of GBM patients, have been shown to return to normal levels after surgery and chemo-radiation, indicating the patient's response to treatment[102]. These miRNAs are glioma-specific, and their loss or downregulation may contribute to tumour progression including malignant transformation of meningioma and pituitary adenomas into GBM [115,116]. A meta-analysis demonstrated that serum miR-125b, markedly reduced in GBM patients, shows a 3.5-fold higher detection frequency compared to healthy controls, highlighting its potential as a screening biomarker[117]. Similarly, miR-205, another tumour suppressor, was significantly decreased in GBM patients, increased post-surgery, and declined again upon recurrence, suggesting its value as a dynamic biomarker[107]. Accordingly, the dysregulated expression of miRNA, shapes the prediction of cancer, early diagnosis, prognosis and histological classification[9,98]

Other studies have advocated that the BBB is not permeable for some miRNAs which increases the value of miRNA profiling of the CSF samples. As miR-10b is greatly overexpressed in GBM but absent in normal brain, it can thus be detected in CSF of majority of GBM patients (89%) but not in the CSF of healthy controls. Considering extracranial tissues express miR-10b, its absence in CSF of healthy controls designated that it may not pass the BBB under non-neoplastic conditions[98,118].

Several studies have identified specific miRNAs that are dysregulated in GBM patients, with both upregulated and downregulated miRNAs linked to tumour progression and prognosis. miRNAs such as miR-21, miR-128, and miR-342-3p have been highlighted for their potential as biomarkers for early GBM detection, offering high sensitivity and specificity, particularly in blood and urinary samples. These clinical data signify that the dysregulation of miRNAs, whether through upregulation or downregulation, can be used to diagnose or monitor GBM patients. The circulation biomarker: miRNA should serve as a non-invasive method for screening patients suspecting GBM. Though the results are thought-provoking, some limitations do exist, like the small size of regimens and the lack of a standard method for blood collection, RNA extraction and sequencing. Of note is that specificity of miRNA detection may be lower than that of ctDNA[9], but it may be considered complementary to ctDNA detection. Despite challenges, miRNA-based liquid biopsy offers a promising, less-invasive alternative to traditional tissue biopsies for GBM diagnosis, prognosis, and treatment monitoring.

Table 4. miRNA isolation and analysis in patients with GBM.

Isolation method	Patient numbers	Markers used to verify GBM origin	Findings	Reference and publication date
miRNA extraction and qPCR	20	miR-221 and miR-222	Both miR-221 and miR-222 were significantly upregulated in the plasma of GBM patients compared to healthy controls. miR-221 demonstrated 90% sensitivity and 100% specificity and miR-222 demonstrated	[119] (2019)

		85% sensitivity and 100% specificity for GBM detection. Expression levels of miR-221 and miR-222 decreased following treatment	
miRNA profiling performed using the **Nanostring®** **platform**	91	**miR-223** and **miR-320e, IDH mutation status** and **1p/19q co-deletion.**	Dynamic changes of miR-320e were linked to tumour volume of GBM patients. A **9-miRNA signature** was established, distinguishing glioma patients from healthy controls with **99.8% accuracy.** miRNA levels did not increase in cases of pseudo-progression. This supports their use in distinguishing true progression from treatment effects and in post-operative monitoring	[120] (2020)
mirVana™ **miRNA Isolation** **Kit** and qRT-PCR	50	**miR-21**, **miR-128**, and **miR-342-3p**	**miR-21 was significantly upregulated,** while **miR-128 and miR-342-3p were markedly downregulated** in glioma patients compared to healthy controls Notably, miR-21 demonstrated a high diagnostic performance with **90% sensitivity**, and **100% specificity**.	[101] (2012)
RNA isolation and NGS, including mRNA-seq and small RNA-seq	7	mRNA and miRNA candidates	The study identified differentially expressed genes in individual patients, with up to 93 mRNA and 19 miRNA candidates linked to GBM recurrence.	[121] (2024)
Urinary microRNA-based diagnostic model for CNS tumours using nanowire scaffolds.	119	Differential miRNA expression profiles	The study reported high diagnostic performance, with sensitivity and specificity values of 100% and 97%, respectively, for detecting early-stage CNS tumours. Non-invasive method holds promise for early detection and monitoring of CNS tumours through urine-based liquid biopsy.	[122] (2021)
miRCURY RNA Isolation Kit and qRT-PCR	10	miR-21, miR-218, miR-193b, miR-331, and miR-374a, miR-548c, miR-520f, miR-27b, and miR-130b	Sampling of CSF from the lumbar region to extract 9 signature miRNA for GBM. The overexpressed signatures were miR-21, miR-218, miR-193b, miR-331, and miR-374a, while the down regulated were miR-548c, miR-520f, miR-27b, and miR-130b in GBM CSF The study compared the diagnostic performance of miRNA detection between CSF obtained from the cisternal and lumbar regions. The cisternal CSF samples demonstrated a sensitivity of 80% and specificity of 67% for GBM detection, whereas the lumbar CSF samples showed a sensitivity of 28% and specificity of 95%.	[123] (2017)
TaqMan Low Density Array platform for miRNA profiling and qRT-PCR	16 GBM and 9 healthy patients	**miR-451**, **miR-711**, **miR-935**, **miR-223**	Showed that miRNA from CSF can differentiate between tumour and non-tumour diseases states. Identified distinct miRNA signature in CSF that can distinguish CNS malignancies (GBM) from non-tumour controls.	[124] (2015)

2.5. Extracellular Vesicles

Extracellular vesicles (EVs), including exosomes and microvesicles (MVs) are small membrane enclosed particles released by normal and tumour cells. They serve as carriers of macromolecules in liquid biopsies of brain tumour patients[18]. EVs are heterogeneous in size, quantity and origin of

molecular content and biological activity. Two main groups of EVs exist, which differ in origin and size. MVs (50 to 500 nm) bud from the cell membrane, while exosomes (50 to 150 nm) originate from the endosomal system[125,126]. EVs protect their cargo; mRNAs, miRNAs, lipids or proteins, which are specific to the cell origin[127] from enzymatic degradation via their lipid bilayer, enabling them to cross the BBB[125].

The significance of EVs is emphasised by the fact that their transcriptomic and proteomic profile is specific to the cell of origin and can differ in response to diverse stimuli[10]. Their imperative role in intercellular communications allows them to alter the phenotype of recipient cells by transferring genetic information and proteins[12,128]. These molecules often reflect malignant processes, underlining their importance as liquid biopsy tools in cancer, including GBM[127]. EV-mediated crosstalk has been demonstrated between GBM and its tumour microenvironment, fostering tumour progression[129]. Exosomes from hypoxic GBM cells overexpress VEGF-A, disrupting the BBB by downregulating occludin and claudin-5[130].

EV isolation typically involves immunoaffinity capture or differential centrifugation gradients[9]. Exosomes are characterised by transmission electron microscopy (TEM), nanoparticle-tracking analysis (NTA) and surface markers such as CD63, CD81, CD9, CD37, CD53, CD82, ICAM-1 and integrins, detectable by flow cytometry or Western blot[127].

Exosome-based liquid biopsies show promise in cancer diagnostics, with tests like ExoDx™ Lung (ALK) and ExoDx Prostate IntelliScore (EPI) already approved. For GBM, research is ongoing[131]. Emerging biomarkers include exosomal circular RNAs (circRNAs), EGFRvIII RNA for monitoring CAR-T cell therapy response, and MGMT methylation for predicting treatment outcomes. Despite promising findings (see Table 5), no exosome-based GBM test has yet reached clinical practice, pending further validation in larger cohorts [131–135].

Studies indicate that EVs from GBM stem cells exhibit increased adhesion-related proteins after TMZ treatment, potentially promoting tumour progression[136]. TMZ resistance may be associated with EVs carrying MGMT mRNA, as a potential marker of resistance, alongside adhesion proteins (e.g., TGM2, CD44 and CD133), and stemness markers like NESTIN[137–139]. These EV-associated molecules may serve as biomarkers to monitor drug resistance and treatment failure[10].

Overall, EVs, particularly exosomes, are promising biomarkers for GBM detection, monitoring, and prognosis. Exosomal RNA, proteins, and lipids include disease-specific molecular cargo, such as EGFRvIII RNA and syndecan-1 with high diagnostic potential. EV-derived molecular cargo may reflect or potentially even contribute to treatment resistance and tumour progression, though their mechanistic role remains under investigation. Although exosome-based liquid biopsy presents a minimally invasive alternative to traditional tissue biopsies, larger patient cohorts need to be analysed and standardised isolation and characterisation techniques are needed to validate their utility in GBM. Despite these limitations, ongoing research suggests that EV-based liquid biopsies could revolutionise GBM management, offering real-time insights into disease progression and therapeutic response.

Table 5. EV isolation and analysis in patients with GBM.

Isolation method	Patient numbers	Markers used to verify GBM origin	Findings	References and publication date
Differential centrifugation and flow cytometry	11	GFAP	Concentration and composition of circulating MVs in patient plasma correlated with tumour progression. Elevated levels of tumour-derived MVs were associated with true tumour progression, while lower levels were indicative of treatment-related changes or pseudoprogression.	[140] (2014)
Differential centrifugation and ultracentrifugation,	25	Tumour-specific EGFRvIII mRNA within the vesicles	EVs contain functional RNA and proteins that may influence the tumour microenvironment and serve as diagnostic tools	[141] (2008)

Method	n	Biomarker	Findings	Ref (Year)
filtration and flotation density gradient centrifugation.			GBM cells release MVs containing mRNA, miRNA, and angiogenic proteins. Detection of tumour-specific EGFRvIII mRNA in serum-derived MVs supports their potential as non-invasive biomarkers for GBM diagnosis and monitoring	
Serial centrifugation and flow cytometry	16	**Annexin V**, **CD41**, **CD235** and **Anti-EGFR**	An increase in Annexin V-positive MV levels during chemoradiation therapy was associated with earlier tumour recurrence and shorter overall survival. Patients with higher levels of MVs had > 4-fold increase in the hazard ratio for recurrence compared to those with lower levels. The study provided initial evidence that monitoring blood-borne MV levels could serve as a non-invasive method to predict disease progression and patient outcomes in newly diagnosed GBM patients.	[142] (2016)
Ultrafiltration and ultracentrifugation and NTA	43	GFAP	Plasma EV concentration was higher in GBM compared with healthy controls (p=0.0099). Average size of GBM and healthy EVs were similar in discovery (p=0.548) and validation cohort (p=0.075). The amount of circulating EV was not affected by tumour size (p=0.318). However, the extent of necrosis influenced the degree of secretion (p=0.045): higher necrosis in GBM samples (grade 3) substantially reduced EV secretion. Elevated EV levels in GBM plasma decrease post-surgery and rose at recurrence.	[143] (2019)
Precipitation using ThermoFisher kit and Semi quantitative RT-PCR	96	EGFRvIII mRNA within the vesicles	EGFRvIII prevalence in the data set was 39.58%. The sensitivity and specificity of serum EV analysis for EGFRvIII was 81.58% (95% CI 65.67%–92.96%) and 79.31% (95% CI 66.65%–88.83%), respectively	[144] (2018)
Differential centrifugation and qRT-PCR	60	miR-301a	Serum exosomal miR-301a levels were significantly elevated in glioma patients compared to healthy controls. Higher miR-301a levels were associated with higher tumour grades and lower Karnofsky Performance Status (KPS) scores. Post-surgical samples showed a significant reduction in miR-301a levels, which increased again during tumour recurrence, suggesting its potential as a marker for disease monitoring. Kaplan-Meier survival analysis indicated that patients with higher serum exosomal miR-301a levels had shorter overall survival.	[145] (2018)
Ultracentrifugation and TEM	42	miR-320, miR-574-3p and RNU6-1	RNU6-1 identified in serum exosomes could effectively distinguish GBM patients from healthy individuals. The elevated levels of RNU6-1 in GBM patients' exosomes suggest its potential as a non-invasive diagnostic biomarker.	[146] (2014)
Differential centrifugation and qPCR	12	**miR-182-5p, miR-328-3p, miR-339-5p, miR-340-5p, miR-485-3p, miR-486-5p** and **miR-543**	The identified miRNA signature in serum exosomes could effectively distinguish GBM patients from healthy individuals and those with lower-grade gliomas.	[99] (2018)
Density gradient ultracentrifugation, using OptiPrep™ Density Gradient Medium and sequencing followed	12 GBM (astrocytoma grade IV) and 5 astrocytoma grade II-III	Analysis of CUSA (cavitron ultrasonic surgical aspirate) EV and serum EV miRNA and piRNA	Seven miRNA species were determined to be the most stable classifiers for GBM (miR-182-5p, 382-3p, 339-5p, 340-5p, 485-3p, 486-3p and 543) with an overall predictive power of 91.7%; within multivariate models, six iterations of these markers were capable of distinguishing GBM patients from healthy controls with 100% accuracy.	[100] (2020)

by differential expression analysis				
Chemical precipitation using ExoQuick-TC and qPCR	100 patients with glioma, 11 with metastatic brain tumours, 30 healthy patients	Expression of 3 miRNAs: miR-21, miR-222 and miR-124-3p, in serum exosomes.	Exosome miR-21, 222 and 124-3p were robust for discriminating patients with GBM from healthy controls, with an area under curve of 0.84 (95% CI 0.7538–0.9371, p < 0.001), 0.80 (95% CI 0.6967–0.8980, p < 0.001) and 0.78 (95% CI 0.6732–0.8904, p < 0.001), respectively. miR-21 alone appears to be the best predictor for distinguishing patients with High grade glioma from those with low grade glioma, displaying the highest AUC of 0.83 (95% CI 0.7395–0.9398, p < 0.001	[147] (2018)
Ultracentrifugation, NTA and TEM for plEV isolation with proximity extension assay–based ultrasensitive immunoprofiling.	82	Syndecan-1	SDC1 in plEVs could discriminate between GBM and low-grade glioma with a sensitivity of 71%, and specificity of 91%. The findings support the concept of circulating plEVs as a tool for non-invasive diagnosis and monitoring of gliomas.	[148] (2019)
Total Exosome Isolation reagent followed by ultracentrifugation and qRT-PCR	43 GBM, 23 other brain tumour patients and 40 healthy individuals	Serum analysed for presence of EVs and HOTAIR biomarker	HOTAIR can be used as a biomarker for Dx and progression of some brain tumours including GBM with sensitivity and specificity of HOTAIR 86.1% and 87.5% respectively.	[149] (2018)
Serial ultracentrifugation and short non-coding RNA sequencing using the OASIS-2.0 platform.	5	Isolate EV's from human differentiated GBM cells in vitro and perform short, non-coding RNA sequencing to determine expression pattern	Small genome sequencing revealed a total of 712 non-coding RNA sequences most of which have not been associated with GBM EV's previously, including the let-7 miRNA family, miR-3182, miR-4448, miR-100-5p and miR-27-3p. Furthermore, multiple non-microRNA short non-coding RNA species were identified including piRNA, snRNA, snoRNA, and yRNA.	[150] (2020)
qPCR	5 Glioma patients before and after radiotherapy	Expression signature of miRNAs in glioma patients before and after radiotherapy	Identified 18 up-regulated differentially expressed (DE) miRNAs and 16 down-regulated DE miRNAs and the target genes of DE miRNAs were predicted based on multiple miRNA-target databases.	[151] (2020)
Differential centrifugation and DNA analysis through methylation array analysis Proteome analysis with differential quantitative proteomics	Unspecified number of glioma patients and non-tumorous temporal tissue from patients undergoing epilepsy surgery	DNA and protein analysis of Glioma and non-tumorous EVs	Tumour-specific mutations and copy number variations were detected in EV-DNA with high accuracy. Proteome analysis did not allow specific tumour identification or classification	[152] (2021)
Size-exclusion chromatography (by using qEV columns from IZON®), followed by immunoprecipitation with CD44-conjugated beads and qRT-PCR	55	Suitability of novel EV isolation procedure and analysis of serum EVs for miRNA biomarkers and their correlation with prognosis.	Four serum biomarkers were identified to be predictive of prognosis, miR-15b-3p, miR-21-3p, and miR-328-3p exhibiting a negative correlation (high levels were associated with an inferior prognosis) and miR-106a-5p a positive correlation (high levels were associated with a better prognosis prediction of GBM prognosis	[153] (2020)

Differential centrifugation and NTA	96 total patients, 24 GBM, 24 meningioma, 24 BM from NSCLC and 24 controls from patients with benign disk herniation.	Proteomic analysis of serum and serum derived small EVs	Identified 10- and 17-membered protein panel for whole serum and sEV samples respectively. While none of these proteins appeared to be able to distinguish between the patient groups individually, their combination was found to reliably discriminate between the different patient groups suggesting that instead of a few candidates, a specific protein panel is required for a perfect differentiation between various tumour types.	[154] (2020)
Ultracentrifugation and RNA extraction from EVs, ddPCR and flow cytometry for rare mutation detection.	14 glioma patients (4 with GBM)	Mutant IDH1 G395A	CSF is a viable bio fluid to examine contents of EVs. Mutant IDH1 G395A identified in CSF EVs with a sensitivity of 63% and specificity of 100%. CSF EVs generally contained higher levels of mutant mRNA than serum EVs. EVs carry tumour-specific RNA signatures that can be detected non-invasively, supporting the potential of EVs as liquid biopsy tools for glioma diagnosis and monitoring.	[155] (2013)
Ultracentrifugation and purification with sucrose cushion for EV isolation. qRT-PCR for mRNA transcript detection.	25 GBM, 5 low-grade glioma and 4 healthy patients	EGFRvIII mutant mRNA	EGFRvIII oncogene, in EV RNA can be accomplished with a sensitivity of 60% and 98% specificity in comparison to the gold standard qPCR of EGFRvIII transcript from brain tumour tissue.	[156] (2017)
Ultracentrifugation and. TEM for EV isolation. Western blotting for exosomal markers. qRT-PCR for mRNA transcript quantification.	9 GBM and 5 healthy patients	Exosomal markers (CD63 and TSG101) miR-21	Showed that miR-21 from CSF EVs can differentiate between tumour and non-tumour diseases states.	[139] (2013)
Ultracentrifugation and **OptiPrep™ density gradient ultracentrifugation for further purification. NTA, flow cytometry and western blotting (to detect PD-L1 on EV surface).**	10	Patient derived GBM stem cells.	PD-L1 expression on the surface of GBM derived EVs, can prevent T-cell activation and proliferation upon binding directly to PD-L1. This indicates PD-L1 expression on EVs can be an immune-escape mechanism for GBM	[157] (2018)
Differential centrifugation of MVs and microfluidic chip-based immunomagnetic technique called µNMR (miniaturized nuclear magnetic resonance) for protein typing of EVs/MVs. Also used magnetic nanoparticles (MNPs) conjugated to antibodies to detect specific tumour-	15	EGFRvIII mutant, PDPN and IDH1	**Tumour-derived MVs carrying EGFR/EGFRvIII proteins were successfully detected in patient plasma.** Showed four protein panels of EV surface proteins can be used to discriminate GBM patients from healthy controls using novel antibody capture method. The system enabled **real-time monitoring of tumour progression and treatment response** by tracking changes in circulating tumour-derived MV profiles.	[158] (2012)

Method	Sample size	Biomarkers	Findings	Reference (Year)
associated proteins on circulating MVs.				
Ultracentrifugation for exosome isolation and characterisation by western blotting for expression protein markers, TEM for morphology and NTA for size and concentration.	10	CD63, CD81 and CD9	Saliva and plasma were used as liquid biopsies to isolate exosomes before and after surgery. GBM patients exhibited smaller but more abundant exosomes compared to healthy donors. EVs from GBM plasma showed **globally reduced levels of cytokines and co-stimulatory molecules**, but **PD-L1 presence** was similar to healthy donors.	[159] (2020)
Proteomics using mass spectrometry	22	MGMT and IDH statuses and GAP43	CSF proteomics using mass spectrometry enables GBM biomarker discovery from small volumes (~30 μL). Mikolajewics et al., identified 755 unique proteins in 73 CSF samples (22 GBM), with MGMT and IDH statuses accurately detected at 94.1% and 33.3%, respectively. Single-cell RNA sequencing confirmed GAP43 as GBM-specific, while TFF3 and CACNA2D2 were specific to BM and CNS lymphoma.	[160] (2022)
Proteomics with sequential window acquisition of all theoretical fragment ion spectra mass spectrometry (SWATH-MS) and immunohistochemistry for validation.	134	BCAS1, INF1, and FBXO2	Identified overexpressed proteins CSF proteomics in recurrent GBM, to quantify the proteomes of newly diagnosed and recurrent GBM patients and validated the markers using immunohistochemistry.	[161] (2023)
Lipidomics using Quadrupole time-of-flight liquid mass spectrometer Q-TOF LCMS/MS	14 GBM and 14 healthy patients	NA; based on **statistically significant differences** in blood lipid species between GBM and controls	Lipidomics, also holds potential Identified differential lipid species including fatty acids, saccharolipid, sphingolipid, glycerolipid and sterol lipid from blood samples.	[162] (2022)

3. Application of Machine Learning and Artificial Intelligence

The diagnosis of glioblastoma using signals derived from liquid biopsy samples remains particularly challenging, traditional statistical approaches frequently fall short in detecting the nonlinear patterns and subtle molecular variations that characterize GBM biology, increasing the risk of misdiagnosis or missed detection when relying solely on liquid biopsy data. To address these challenges, artificial intelligence (AI) and machine learning (ML) approaches are increasingly integrated into GBM liquid biopsy workflows. Novel ML algorithms can extract meaningful insights from noisy and heterogeneous datasets, enabling earlier and more accurate detection of GBM tumours, even when biomarker levels are extremely low in blood or CSF. Various liquid biopsy-based signatures have shown potential for tumour detection, including plasma denaturation profiles, CSF proteomic signatures, and serum miRNA signatures120, 160. In omics analysis for GBM, ML techniques uncover complex molecular signatures linked to tumour subtype classification, disease progression, and therapeutic response[163,164]. This allows the development of robust predictive models that outperform traditional biomarker-based approaches, advancing precision diagnostics and personalized treatment strategies for glioblastoma patients. Once trained, these models can be applied in clinical practice[163,164].

Fragmentation patterns and personalised cfDNA sequencing in urine and plasma have differentiated glioma patients from healthy individuals[165]. Tumour-educated platelets (TEPs),

analysed with swarm algorithms, have identified spliced RNA biomarkers to distinguish false positives from true disease progression[166].

ML has also identified tumour-specific DNA methylation markers in blood and tumour-associated MRI features[167,168]. In several studies, ML algorithm identified MRI based tumour-associated features, while serum spectroscopy combined with MRI detected spectra variations between healthy and tumour-affected patients. Additionally, ML has been applied to investigate the relationship between brain tumour volume and liquid biopsy test performance. In a cohort of 177 patients (90 patients with high-grade glioma (GBM or anaplastic astrocytoma), or low-grade glioma (astrocytoma, oligoastrocytoma and oligodendroglioma) spectroscopic liquid biopsy approach detected small and low-grade gliomas, supporting early diagnosis[169,170].

AI enhances liquid biopsy applications and diagnostic performance by enabling rapid analysis of cancer-related circulating proteins and nucleic acids and enhancing imaging from subjective interpretation to quantitative analysis.[171–173] Multimodal AI models integrate proteomic and genomic data from liquid biopsies and quantitative imaging to create a more comprehensive diagnostic pathway[174,175]. This helps distinguish benign from malignant lesions, reduces unnecessary follow-ups and guides therapeutic decisions.

Despite its potential, the application of AI and ML in liquid biopsy and cancer diagnostics faces several challenges. One major hurdle is the need for large, high-quality datasets to train robust models, as variability in sample collection, processing, and sequencing methods introduces bias[176]. Additionally, the interpretability of AI-driven models remains a concern, as complex algorithms often function as "black boxes," making it difficult for clinicians to understand the reasoning behind predictions[164,174]. Regulatory approval and clinical validation also pose significant barriers, requiring extensive trials to ensure reliability and accuracy. Finally, integrating AI into existing healthcare systems demands substantial computational resources, infrastructure, and clinician training, which may limit widespread adoption[168,174,176–178].

4. Conclusions

In the coming years, clinical studies are expected to clarify which liquid biopsy techniques provide the most value for diagnosing, prognosing, and treating primary brain tumours, potentially surpassing traditional imaging and tissue-based methods. GBM liquid biopsy research is rapidly gaining traction. Analysis of body fluids, such as blood, or CSF, from GBM patients reveal various tumour-derived components, including CTCs, ctDNA, miRNAs, EVs, nucleosomes, and metabolites. These biomarkers may serve as alternatives to tissue samples and support genotype-guided therapies and personalised GBM management. They also show promise for monitoring tumour progression, treatment response, and guiding therapeutic choices. However, accessing tumour-derived material is challenging due to the BBB, which limits the release of biomarkers into the bloodstream—currently the most studied liquid biopsy. Consequently, tumour-derived nucleic acids in serum or plasma often occur at low levels, hindering routine clinical use. Improving sensitivity will require optimising sample volumes, technological platforms, and the use of artificial intelligence. Combining the measurement of multiple different liquid biopsy-based markers from the same biopsy will likely improve overall sensitivity of single modality testing but this idea needs to be further validated in the future.

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