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Time to focus on circulating nucleic acids for diagnosis and monitoring of gliomas: a systematic review of their role as biomarkers

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Abstract

Gliomas are diffusely growing tumours arising from progenitors within the central nervous system. They encompass a range of different molecular types and subtypes, many of which have a well-defined profile of driver mutations, copy number changes, and DNA methylation patterns. A majority of gliomas will require surgical intervention to relieve raised intracranial pressure and reduce tumour burden. A proportion of tumours, however, are located in neurologically sensitive areas and a biopsy poses a significant risk of a deficit. A majority of gliomas recur after surgery, and monitoring tumour burden of the recurrence is currently achieved by imaging. However, most imaging modalities have limitations in assessing tumour burden and infiltration into adjacent brain, and sometimes imaging is

unable to discriminate between tumour recurrence and pseudo-progression. Liquid biopsies, obtained from body fluids such as cerebrospinal fluid or blood, contain circulating nucleic acids or extracellular vesicles containing tumour-derived components. The studies for this systematic review were selected according to PRISMA criteria, and suggest that the detection of circulating tumour-derived nucleic acids holds great promises as biomarker to aid diagnosis and prognostication by monitoring tumour progression, and thus can be considered a pathway towards personalized medicine

Keywords: Glioma, biomarkers, circulating nucleic acids, CSF, blood, liquid biopsy.

Abbreviations

Glioblastoma multiforme (GBM)
World Health Organization (WHO)
Isocitrate dehydrogenase (IDH)
Central Nervous System (CNS)
Magnetic Resonance Imaging (MRI)
cell-free nucleic acids (cfNAs)
microRNAs (miRNAs)
long-non-coding RNAs (lncRNAs)
Cerebrospinal fluid (CSF)
Blood brain barrier (BBB)
cell-free DNA (cfDNA)
Circulating tumour DNA (ctDNA)
Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA)
cell-free RNA (cfRNA)
Progression-Free Survival (PFS)
Disease-Free Survival (DFS)

Overall Survival (OS)

Extracellular vesicles (EVs)

Copy number variations (CNVs)

Next Generation Sequencing (NGS)

REporting recommendations for tumour MARKer prognostic studies (REMARK)

Introduction

Gliomas are the most common intrinsic brain tumours and are thought to arise from progenitor cells in the central nervous system (CNS) [1]. There is a significant variability between and within different tumour types, and a proportion of gliomas, such as the malignant form glioblastoma multiforme (GBM), show a significant degree of intratumoural heterogeneity [1,2]. The discovery of significant driver mutations such as isocitrate dehydrogenase (IDH) in 2008, BRAF, or histone K27M has fundamentally changed the diagnostic approach in neuropathology [3-5]. This biomarker-driven classification is reflected in the 2016 update of the World Health Organization (WHO) classification of CNS tumours. This has resulted in the concept of an integrated diagnosis, combining histological diagnosis and molecular profile [6-8]. The diagnostic approach starts with the histological examination, followed by immunohistochemical stains and subsequent genetic or epigenetic analysis, to determine a mutation defining a tumour type or even to establish a methylation class [6,9]. This diagnostic workup requires tumour material, e.g., a brain biopsy, which can be limited (for example stereotactic needle biopsies), but can also be extensive after a debulking. After surgery, disease is monitored by magnetic resonance imaging (MRI). In patients with adjuvant radio- and chemotherapy, it may be difficult to reliably discriminate tumour progression from radiation necrosis [10]. Whilst tissue biopsies are essential to establish a diagnosis and a molecular profile, they will always represent a static snapshot in time, which cannot reflect changes in the mutational spectrum, microenvironment and heterogeneity during recurrence and progression. The discovery of nucleic acids derived from tumours circulating in body fluids has opened up significant potential for monitoring tumour recurrence, progression and potentially even preoperative diagnosis. The minimally invasive nature of blood sampling facilitates serial testing and the monitoring of dynamic changes during tumour therapy [10]. Liquid biopsy detects molecular contents such as proteins or cell-free nucleic acids (cfNAs) from tumours in body fluids, either as cell-free entities, attached to lipid or protein structures or as the content of vesicles, such as exosomes. Several classes of cfNAs exist: DNA and different classes of RNA, such as protein-coding mRNA and non-coding RNAs, e.g., microRNAs (miRNAs) and long non-coding RNAs (lncRNAs). Cell-free DNA (cfDNA) includes DNA released from cancer cells, bearing tumour-specific genetic alterations and referred to as circulating tumour DNA (ctDNA) [11-14]. Although most liquid biopsies are derived from blood samples, other biofluids, including

cerebrospinal fluid (CSF) can be used [15]. CSF could play a particular role as a source of brain tumour specific biomarkers, as it circulates in close contact with the CNS, and even if its collection requires a lumbar puncture, which is more invasive and fraught with more risks than taking a blood sample, is still considerably less invasive than a brain biopsy.

The role of the blood brain barrier (BBB) in the context of biomarkers derived from brain tumours has been a matter of significant debate. It is assumed that the release of circulating biomarkers requires crossing of the BBB, and it is hypothesized that the scarcity of circulating markers in CSF or blood is due to the BBB [16]. However, a majority of diffuse gliomas, in particular GBM, almost invariably have a disrupted BBB, and a more generous release of circulating biomarkers would be expected [10].

This review aims to discuss and summarize the current literature on cfNAs in blood and CSF of glioma patients. We focus on DNA and RNA (miRNAs and lncRNA) as a useful source of information that might complement the histopathological, molecular, and imaging techniques, and on how they could be adopted into clinical practice for personalised medicine.

Methods

Literature search Strategy

A systematic literature search was performed following the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines in three databases (PubMed, EMBASE and CENTRAL) from January 2000 to March 2020. A search strategy, limited to Title/Abstract, was defined. We used a combination of keywords and Boolean operators for the following key concepts: "circulating microRNAs", "circulating tumour DNA", "cell-free nucleic acids", "glioma", "liquid biopsy". Details of the search strategy in the Supplementary material.

Eligibility criteria

Three co-authors (ABDM, ET, GR) independently screened titles, abstracts and full-text articles according to the following inclusion criteria: (1) full articles in English; (2) articles concerning the analysis of cfNAs in human blood and CSF from glioma patients; (3) studies evaluating nucleic acids as biomarkers. Case reports and review articles were excluded but their bibliography was screened to identify further articles that may have been missed with the search strategy.

Results

Selected studies

After full-text screening, 89 articles were included (**Figure 1A**). One relevant study lacking our keywords and published after completion of the screening was added [17]. All the eligible studies were assessed for quality by the QUADAS-2 tool [18] **(Supplementary material)** and passed the criteria **(Figure S1)**. The highest risk to the quality arose from the index test due to a high variance between the technical methods and normalisers between different studies. Articles regarding circulating miRNAs were homogeneous in terms of the methodology used for detection. Instead, articles about cfDNA were mostly heterogeneous in terms of study design and methodology, rendering them less comparable.

Among the selected citations, 64 articles focused on the analysis of either cell-free RNA (cfRNA) as a single species, or exosomal RNAs **(Table 1; Figure 1B)**, comprising mainly miRNAs [15,19-71] and lncRNAs [72-78], and only few other RNA species [27,79-81]. The other 25 articles discussed cfDNA [82-106] **(Table 2; Figure 1B)**. Articles describing the prognostic impact of cfNAs were selected only if they included Progression-Free Survival (PFS), Disease-Free Survival (DFS), and/or Overall Survival (OS).

Circulating RNA in the context of gliomas

The majority of the selected studies on cfRNA evaluated miRNAs, small non-coding RNAs considered potential circulating biomarkers [15,19-71]. In contrast, only few articles evaluated other RNAs species [27,72-81] such as non-coding single stranded RNAs longer than 200bp (lncRNAs) with important roles in multiple cellular functions [72-78]. However, most of cfRNAs were described in blood [19,21-23,25-37,39-42,44-54, 56-81] and only a few articles focused on CSF [15,20,24,34,35,43,55]. The majority of cfRNA studies were done on patients with GBM (46%), followed by those with diffuse and anaplastic astrocytoma (12% each), whilst 15% of the articles did not specify the glioma subtype **(Figure S2)**.

Cell-free RNA in blood

Of the 46 publications about circulating miRNAs in plasma or serum, 45 described deregulation of miRNAs in glioma patients and compared them to either healthy individuals or patients with

pathologies other than gliomas [19,21-23,25-33,36,37,39-42,44,45,47-54,57-71]. This suggests their potential as diagnostic biomarkers **(Table 1)**. Across these studies, 82 miRNAs were evaluated but only 12 were analysed in samples with homogeneous clinical features and were consistently deregulated [19,21-23,26,28-30,33,36,37,40,42,47,48,50,54,58,63,68-70] **(Figure 2A)**. Among these, miR-21 was the most commonly investigated biomarker [19,21,22,36,37,48,68] and was one of the first miRNAs to be identified as an oncomiR in cancer, including gliomas [21]. miR-21 was upregulated in glioma patients and correlated with higher grades [19,21,22,36,37,48,68]. Another relevant miRNA is miR-497, downregulated not only in glioma tissues and cell lines [104] but also in serum [23,41], and has been reported to correlate with tumour malignancy and to discriminate gliomas from other brain tumours [41].

Several studies explored the correlation of circulating miRNAs with the histopathological grade of glioma [22,23,28,30,32,33,35,39,41,42,44,45,47,49,52,56,63,64,66,69]. However, this approach is fraught with inaccuracies, as it is increasingly recognized that the histological grade may not always accurately reflect the biological behaviour [6]. Yet, only few studies took the opportunity to integrate the molecular profile of gliomas with circulating miRNA levels [54,61,62,67] **(Figure 3)**. Most of them just evaluated the correlation between *IDH* status and the miRNAs of interest [61,62,67]. In the study of Ebrahimkhani *et al.* [54], a 13- miRNA-signature was found to differentiate patients based on *IDH* mutational status with an estimated predictive power of 77.4%, demonstrating the potential of miRNAs for glioma molecular subtyping. Although these studies were performed to provide a diagnostic tool, they may also serve the purpose of identifying the prognostic and predictive value of miRNAs [21-23,25,26,28,29,32,35,37,39,40,46,48,49,51-53,56,57,59-64,66,67] **(Table 1)**.

Overall, 28 circulating miRNAs have been proposed as prognostic biomarkers **(Table 1)**. However, only miR-221, -222, -210, -106a, and -145, were considered in more than one article and were reported as consistently dysregulated [26,28,29,42,52,67,70]. Levels of miR-221, miR-222 [26,42,52], miR-210 [29,70] and miR-106a [28,46] correlate with poorer PFS, OS or 2-year DFS. miR-210 and miR-106a are associated with tumour hypoxia and invasiveness and thus are considered oncomiRs in gliomas [28,29]. In contrast, miR-145 levels are inversely correlated with OS [46,67] and 2 year DFS [46]. This miRNA has also been evaluated as serum diagnostic marker but with inconsistent results [47,67].

Table 1 shows miRNAs that have been identified as potentially useful biomarkers to predict treatment response. miR-128 increases post operatively and during chemo-radiation therapy, and is the only biomarker reported in more than one article with consistent results. [22,30]. Other circulating miRNAs responded to recurrence [45,59,70] and/or therapy response [22,37,59] and were considered as non-invasive predictive biomarkers. For example, deregulation of miR-205 [45] or miR-1238 [59] in serum is related to GBM recurrence and temozolomide (TMZ) resistance. miR-21 was downregulated after surgery and chemo-radiation [22] and upregulated after treatment with bevacizumab [37]. At present, however, these data are either supported by a single study only, or are discrepant between studies, questioning the reliability of these miRNAs as biomarkers.

Seven studies reported on cell-free lncRNAs [72-78] **(Figure 1A)**. In these articles the lncRNAs LINK-A, HOTAIR, GAS5, GASL1, SBF2-AS1, TUSC7 and MIR210HG were considered as potential diagnostic, prognostic or monitoring markers **(Table 1)**. However, among them, only HOTAIR, a well characterized *trans*-acting lncRNA involved in the genome-wide reprogramming of chromatin in glioma [75], was evaluated as biomarker in more than one study [74,75]. Tan and colleagues found HOTAIR elevated in both, total serum and in the exosome-enriched serum fraction from GBM patients, and its reduction two weeks after surgery [75]. Increased HOTAIR levels were associated with poorer survival [75]. A single report shows deregulation of LINK-A, GASL1, GAS5, TUSL7 and MIR210HG in the serum of glioma patients, with varied correlations with prognosis [72-74,76-78]. Specifically, higher levels of LINK-A and MIR210HG are found in glioma patients and overexpression of GAS5 correlated with poorer outcome [72,76]. Conversely, GASL1 and TUSL7 are downregulated in patients and this correlates with poorer prognosis [73,77]. Moreover, only MIR210HG and TUSL7 also correlated with glioma malignancy grade [72,73] **(Figure 3)**. SBF2-ASL1 is the only lncRNA studied in relation to treatment response and is studied in the exosome-enriched fraction [78].

Cell-free RNAs in CSF

Even though the number of studies on cfRNAs in CSF is much smaller than those on blood, we still consider them highly relevant **(Figure 2A)**. Three articles comparing miRNA levels both in CSF and blood are particularly significant [24,34,35]. Shi *et al.* showed that miR-21 in CSF, but not in serum, can discriminate GBM patients from healthy subjects [35], and another study confirms deregulation in

CSF but not in plasma of GBM patients compared with healthy individuals [34]. Whilst CSF contains fewer miRNAs than plasma or serum, it is arguably a more relevant source for brain tumour biomarkers, as blood may contain miRNAs from a broader range of sources and may be more susceptible to "contamination" with hematopoietic cells, potentially hindering detection of relevant miRNAs [34,55]. As previously mentioned, only miR-21 was reported in multiple studies, [15,20,24,35,43] found to be upregulated in correlation with glioma grade [35,48], and it has been evaluated as predictive marker [20,55] or to monitor treatment response [20,35], suggesting that CSF miR-21 is potentially a reliable biomarker for disease monitoring and management, including the assessment of relapses, remissions or efficacy of chemo-radiotherapy [20,35]. For example, evaluation of miR-21 levels showed an increase in a patient 25 weeks post-radiation, correlating with disease progression seen on MRI, PET-CT, and confirmed by tissue biopsy [20]. Other CSF markers, evaluated in single studies only, are miR-15b, miR-10b and miR-193b, were reported as consistently deregulated also in blood [15,20,28,36,37,43,56].

Circulating DNA in glioma patients

These studies, based on the detection of genetic and epigenetic alterations in cfDNA isolated from blood and CSF **(Table 2),** evaluated several genetic alterations as candidate biomarkers including *IDH1* mutation [87-106], copy number variations (CNVs) such as loss of heterozygosity for 1p, 10q, 19q [93,100], gain in chromosome 7 and loss of chromosome 10 [101], EGFRvIII mutation [91] and abnormal methylation of *MGMT*, *p16, DAPK, RASSF1A, p73*, *RARbeta*, *PTEN*, *p15INK4B* and *p14ARF* promoters [82-84] **(Figure 1B)**.

Cell-free DNA in blood

The selected studies on cfDNA in blood comprise studies in plasma [83,86,91,92] and serum [82,84,86,89,90,96,99] **(Figure 2B)**. Most of the studies had as a main objective the analysis of the methylation status of gene promoters or of repetitive elements such as Alu sequences, the most abundant families of repetitive elements of the human genome, frequently hypomethylated in cancer [82,84,85,89,90,96,99,105]. Evidence of a correlation between DNA methylation in glioma tumour samples and in serum ctDNA was provided, for the first time, by Balaña and colleagues [82] who evaluated promoter methylation of *MGMT*, *p16*, *DAPK*, and *RASSF1A* and showed a correlation between *MGMT* methylation and response to treatment and PFS, in tissue and in serum. These data

were confirmed by two other studies [83,85]. Lavon and collaborators [85] found that serum ctDNA in glioma patients is informative for both loss of heterozygosity and DNA methylation status during the course of the disease. The sensitivity was moderate due to false negative samples depending on tumour size, but there was a specificity close to 100% for low- and high-grade tumours [86]. Another study on *MGMT* promoter methylation showed a high concordance between methylation levels in tumour tissue and plasma, and its correlation with longer survival, supporting the utility of the detection of *MGMT* promoter methylation in ctDNA for prediction of treatment response [92]. However, the authors highlighted that only methylated promoter status is specific, whilst the detection of an unmethylated *MGMT* promoter may represent a false negative finding [92]. Three articles discussed the importance of hypomethylation of Alu sequences as biomarker in glioma [90,94,99]. Chen *et al.* [90] showed a statistically significant correlation between Alu methylation level in tumour and serum from all patients. Specifically, they demonstrated that Alu methylation in cfDNA was significantly lower in patients compared to healthy individuals. Surprisingly, Alu methylation levels in cfDNA was lower in patients who died from glioma than in those still alive or that died from other causes. These results indicate that Alu methylation levels may be prognostic in glioma, confirmed by subsequent studies [96].

Gong and colleagues [99] conducted a large study with 124 patients and 58 healthy individuals, and proposed that *MGMT* hypermethylation and Alu hypomethylation could serve as a novel diagnostic and prognostic marker in gliomas. In particular, they showed that Alu sequences were hypomethylated, and *MGMT* hypermethylated in glioma patients, and also p16 methylation levels were altered compared to controls. The methylation levels in tissue and serum correlated, thus supporting the potential use of ctDNA as early diagnostic/predictive biomarker for precision medicine in gliomas. Finally, four studies focused on specific mutations in blood ctDNA [87,91,105,106]. EGFRvIII, a truncated constitutively active mutation in the EGF receptor (EGFR), present in a proportion of *IDH*-wild-type GBMs, was reliably detectable in ctDNA, the study was limited by a small sample size [91]. Bagley *et al.* [106] observed an association of plasma cfDNA levels with PFS and OS and a correlation with radiographic tumour burden, in a cohort of 42 GBM patients. By Next Generation Sequencing (NGS) analysis, they demonstrate the detection of tumour-specific mutations in the serum of 75% of the patients, suggesting that plasma cfDNA has a utility as non-invasive

biomarker of tumour burden, prognosis and may also be a substrate for molecular profiling in association with tissue sequencing [106]. The efficient detection of tumour mutations in plasma ctDNA has also been demonstrated by Piccioni and colleagues [105], who found in a large cohort of brain tumour patients, including those with gliomas, at least one genetic alteration in 50% of patients, with higher proportions when assessing only patients with GBM [105].

Boisselier *et al.* [87] used a combination of COLD (coamplification at lower denaturation temperature) PCR and digital PCR to detect the *IDH1 c.395G>A* (encoding the *IDH1* p.R132H, or $IDH1^{R132H}$) mutation in serum ctDNA. The required specificity of 100% was achieved with a sensitivity of 60% and correlated with the tumour volume measured on MRI [87]. In conclusion, these studies show a correlation of genetic and epigenetic alterations between tissues and blood ctDNA **(Figure 2B)**. However, advancements in MRI techniques and MR spectroscopy which can predict IDH mutation and even 1p/19q codeletion [107] render this assay less useful than a few years ago.

Cell-free DNA in CSF

All studies selected for this review demonstrate the possibility to identify genomic alterations such as CNVs or mutations in CSF with high sensitivity, in many cases comparable to that of tissues or blood analyses, which is of interest for diagnosis and prognostication **(Table 2)**.

Among the selected articles on circulating biomarkers in CSF [86,88,92,94,97,98,100], six reports compared cfDNA levels in both, CSF and blood [86,88,93,94,98,100] **(Figure 2B)**.

One of the first studies on ctDNA in CSF was conducted by Liu *et al.* [86]. These authors used methylated DNA immunoprecipitation to detect gene promoter hypermethylation in *MGMT, p16INK4a, TIMP-3,* and *THBS1* in tumour tissue, serum, and in CSF from glioma patients. They found a correlation between methylation levels and survival, concluding that the methylation status of these genes may become a promising prognostic factor and may serve as minimally invasive tumour marker. Pentasova *et al.* [97] explored DNA profiling by high-throughput sequencing of CSF to evaluate the possibility of identifying tumour-associated mutations in patients with known or suspected brain tumours. Such alterations were detected in patients with tumours but not in healthy individuals, suggesting that characterization of ctDNA in CSF holds promises for disease monitoring [97].

Studies using DNA sequencing showed that target DNA was more readily detectable in CSF than in plasma [94,100]. De Mattos-Arruda [94] showed that CSF ctDNA is more representative than plasma to detect genomic alterations of brain tumours (mutations and CNVs in *EGFR, PTEN, ESR1, IDH1, ERBB2, FGFR2*). Juratli *et al.* [100] conducted a pilot study to assess the feasibility and the potential implications of detecting *TERT* promoter mutations in ctDNA from CSF and plasma of GBM patients. Interestingly, this study, and that of Wang *et al.* [95], showed that the detection of mutations originating from tumour cells in the CSF was greatly facilitated if the tumour was high-grade, and adjacent to CSF cisterns, whilst these markers were not detectable in patients with low-grade gliomas even if in contact to CSF, or when tumours were encapsulated within the brain or spinal cord, suggesting that the tumour location is an important contributing factor to the sensitivity of this assay [95,100]. Martínez-Ricarte *et al.* [103] performed an analysis of seven genes (*IDH1, IDH2, TP53, TERT, ATRX, H3F3A, HIST1H3B*) on 20 tumour specimens and corresponding CSF samples, using a sequencing platform. They demonstrate that genomic analysis of gene mutations in CSF ctDNA allowed the correct classification of 79% of tumours according to the molecular subtype, which can significantly facilitate clinical management of the patients [103]. In a recent publication, Miller and colleagues [104] evaluated the representation of the tumour genome in the CSF of 85 glioma patients. Tumour-derived DNA was detected in the CSF of nearly half of the patients and was associated with disease burden and adverse outcome. The genomic landscape of glioma in CSF demonstrated a broad spectrum of genetic alterations and closely resembled that in tumour samples, suggesting that ctDNA in the CSF may be an early indicator of progression [104]. It has yet to be established if the higher specificity of ctDNA in CSF outweighs the higher risks of lumbar puncture compared with the more accessible serum or plasma and it is conceivable that low-risk procedures may be prioritised (e.g., serum) and in case of non-informative results may be followed up by higher risk interventions (e.g. CSF, brain biopsy).

Extracellular vesicles in glioma

Tumour-derived nucleic acids can be detected as cell-free entities or as the contents of circulating membrane-derived extracellular vesicles (EVs). These are ubiquitous in biofluids including blood and CSF. The most extensively characterised EV categories are microvesicles (200-500 nm diameter) and exosomes (40-100 nm diameter) [108]. Increased microvescicle content has been observed in plasma

from glioma patients suggesting that EVs may represent an important source suitable for profiling of nucleic acids in gliomas [109].

The first record of exosomal non-coding RNA isolated from GBM patients was reported by Manterola and colleagues [27], who found that the expression levels of RNU6-1, miR-320 and miR-574-3p were significantly associated with a diagnosis of GBM. Exosomal miRNAs are involved in several important biological processes, including cell proliferation and resistance to apoptosis [27]. However, amongst all miRNAs evaluated, both in blood and CSF [19,24,27,34,41,48,54,59,61] **(Table 1)** only miR-21 and miR-210 may be considered sufficiently reliable markers, as they have been described in more than one study [41,69,88]. With respect to exosomal lncRNAs, only HOTAIR and SBF2-AS1 have been found as potential markers in our research strategy [75,79]. Tan and colleagues [75] showed that HOTAIR is absent in exosome-depleted serum, suggesting that this lncRNA circulates mainly inside exosomes [75]. Exosomal SBF2-AS1, the only lncRNA studied in the context of chemotherapy response, is increased in GBM patients and associated with poor response to TMZ treatment [79]. However, the debate about the relative contribution of exosomal non-coding RNAs to whole serum/plasma RNA is still open. Although some authors have demonstrated that RNAs in blood exist primarily inside exosomes, where they are detectable at higher sensitivity [75], other studies claim that the majority of non-coding RNAs in blood is located primarily outside exosomes [110]. The reason for these discrepancies is unclear but may be ascribed, at least partly, to differences in isolating exosomes or differences between plasma and serum. Indeed, unlike in plasma, serum miRNAs were more commonly found in exosomes than freely circulating [111].

Other studies have demonstrated that circulating EVs may be a source of material to analyse tumour-specific mutations [19,79,80]. In particular, Chen *et al.* described a novel approach that combines beads, emulsions, amplification, magnetics (BEAMing) RT-PCR and droplet digital PCR to identify glioma mutations. EVs from patient CSF were used to reliably detect and quantify mutant and wildtype IDH1 mRNA [79]. Moreover, RNA from EVs has been used to detect the EGFRvIII transcript in GBM patients [19,80,81]. In particular, in a multicentre study of 71 patients, Figueroa *et al.* demonstrated that CSF-derived EVs may allow the detection of EGFRvIII and *EGFR* amplification [81]. The current difficulties in isolating tumour-specific EVs remain a challenge and may limit their use in clinical settings. For these reasons, several methods have been proposed to

isolate EVs from biofluids [79,80]. A sensitive analytical microfluidic platform (EVHB-Chip) for tumour-specific EV-RNA isolation has achieved 94% tumour-EV specificity, has a detection limit of 100 EVs/μL, and a 10-fold tumour RNA enrichment compared to other methods [80]. Testing serum/plasma samples of GBM patients with the EVHB-Chip, relatively rare EGFRvIII transcripts were identified, as well as genes expressed in specific GBM subtypes [80].

Discussion

Gliomas represent a medical challenge due to their anatomical location, their diffuse and infiltrative growth, the resulting impact on brain functioning, and their biological complexity [1]. The histological and molecular stratification of tumour types is based on multiple genetic and epigenetic characteristics of the tumour that have both diagnostic and prognostic value. In mainstream diagnostic pathology settings, these molecular markers are tested on histologically assessed, and thus validated, starting material. This approach can be limited when patients are at increased risk from a biopsy, due to tumour location, clinical performance status or comorbidities [2]. Liquid biopsies, in contrast to tissue biopsies, have the potential to extend the diagnostic options for high-risk patients, by their minimally invasive approach, for example allowing repeated sampling for disease monitoring. With continuous refinement of technological options, liquid biopsies can potentially assess a wide range of molecular markers, with high sensitivity and specificity. The results may in the future facilitate the clinical decision-making process, to balance the risk and benefits of surgical tumour removal. Initially, liquid biopsy studies focused on circulating tumour cells, but interest soon moved towards cfNAs, as they are easier to collect and analyse [11]. The discovery of cfNAs in the blood stream dates back to the detection in 1947 by Mandel and Metais, of DNA and RNA in the plasma of healthy and ill individuals [12,112]. Forty years later, cancer-specific DNA mutations were found in the blood of cancer patients, suggesting the potential of ctDNA as a tumour biomarker [113]. In 2008, circulating miRNAs were reported for the first time in the serum of glioma patients [19]. cfDNA comprises small fragments of DNA (180–200 base pairs) released by cells under physiological and pathological conditions. It is suggested that the main source of cfDNA are apoptotic cells [12], although active secretion has also been proposed. When released by cells under physiological conditions, these fragments are generally cleared by phagocytosis, thus cfDNA levels are typically low in healthy individuals [13]. In tumours, the amount of cfDNA varies and is thought to reflect the burden of disease [11,85]. cfDNA might carry tumour-specific mutations, as ctDNA, representing an important source of information about the tumour type [14]. Tumour cells can also release different classes of RNA, such as mRNA and non-coding RNAs. Among the latter, miRNAs are the most frequently investigated as biomarkers mainly due to their frequent alteration in cancer [115] and their high stability in biofluids [116,117].

The studies reviewed here mostly focus on cfRNAs, in particular non-coding RNAs. This category of transcripts is frequently deregulated in biofluids of glioma patients and, with a sensitivity in the range of ~30-99 % and a specificity of ~70-100 %, hold promise for disease detection [15,22,24,26-33,35,36,40-44,52,54-56,61-65,67,70,71,75-77]. miR-21, consistently upregulated in patients biofluids, is one of the most investigated miRNAs, and is considered an oncomiR [21]. It is implicated in a variety of cellular and molecular pathways relevant in gliomagenesis [15]. miR-497, another candidate biomarker, is implicated in glioma cell growth and invasion through the Wnt3a/c-jun/miR-497 feedback axis and is associated with angiogenesis and TMZ resistance by targeting mTOR/Bcl-2 [118]. Some miRNAs correlate with patient outcome and can serve as prognostic/predictive biomarkers [21-23,25,26,32,33,35,37,39,40,42,45,46,49-53,56-64,66-68,70], with the most relevant ones belonging to the miR-221/222 family, which is involved in glioma cell invasion, proliferation, and apoptosis, regulating multiple target genes [26]. However, the value of circulating miRNAs as prognostic marker or tool for monitoring tumour growth has been less explored, and in many studies miRNA levels were evaluated just before and after surgery [20,22,23,28,30,39,42,45,48,61,63,68,70] and not in relation to chemotherapy [22,37,59]. Despite the large number of cfRNA molecules examined, only few of them have yielded consistent results across different studies. This high variability limits their utility as non-invasive biomarkers and could be explained by differences in study design, sample size, ethnicity, and methodology used [28]. Another limitation is the lack of an association of cfRNA to the molecular features of the tumours as, despite the 2016 update of the WHO classification, few articles have evaluated the correlations with molecular markers [54,61,62,67].

There are fewer articles studying cfDNA [82-106] in gliomas than those on cfRNA [15,19-81], but the high specificity (>80%) [85,87,89,94,96,99,100] in detecting tumour-derived cfDNA is compelling, rendering them potentially suitable for complementing molecular analysis of tumour biopsies, even if sensitivity levels can be highly variable (8-80%) depending on the biomarker evaluated and the biofluid analysed [85,87,89,94,96,99,100]. The small number of these studies can be explained by the difficulties in retrieving ctDNA from blood of patients with gliomas compared to other cancers [119].

The presence of BBB must be taken into account when considering cfNAs as brain tumour markers. The BBB integrity is often compromised in gliomas with different degrees of disruption in different stages and grades. Changes in BBB permeability can be observed on MRI [16]. To investigate the influence of BBB on circulating biomarkers, Nabavizadeh and colleagues evaluated the relationship between MRI metrics, reflecting BBB disruption, and plasma cfDNA concentration in GBM patients. They found that cfDNA concentration and tumour volume correlated only in patients with high degrees of BBB disruption. These data suggest that the reliability of cfDNA as a biomarker is dependent on the level of BBB permeability [119]. However, how aspects of the BBB impact on liquid biopsy is still unknown, thus more studies are required to address this issue. EVs as carriers of tumour-derived components are of great interest in liquid biopsies in glioma patients, as they can cross the BBB and are readily detectable in the plasma [120]. However, the evaluation of BBB permeability by MRI could be used as a complementary analysis with cfNAs evaluation to support their reliability as biomarkers in gliomas [16].

In conclusion, the studies discussed in this review demonstrate the increasing role and potential of cfNAs as biomarkers that can be obtained with minimal (blood) or moderate (CSF) invasiveness. With increasingly sensitive detection methods and continued validation they may, at some point, become an additional diagnostic tool for detection of alterations that occur early during tumourigenesis and are specific for certain tumour types (for example *IDH* mutation, 1p/19q codeletion, *EGFR* amplification, *TERT* promoter mutation, histone mutation etc), which is useful for stratification of patients with certain glioma subtypes [81,87,91,100, 103,104,106]. This might help with clinical trial stratification and genotype-directed therapies of these tumours. Moreover, cfNAs may be used for early identification and monitoring of relapses, for assessing therapy response of gliomas (e.g., monitoring changes in variant allele fraction of a given mutation) [20,35,59,82,99,105] and potentially also discriminating progression from pseudo-progression, with the possibility of identifying subgroups of patients amenable of alternative therapeutic approaches [106]. The diagnostic implementation will now require a validated detection method in order to improve reliability and clinical utility of the analysis, agreement on the most suitable biomarkers, and ideally prospective clinical biomarker trials. Clinical studies on cfNAs should adhere to REporting recommendations for tumour MARKer prognostic studies (REMARK) guidelines [121]. For the time

being, multiple avenues need to be pursued to identify the most suitable methodologies, biomarkers and test indications.

Conflict of interest statement

The authors disclose no financial and personal relationships with other people or organizations that could inappropriately influence the article content.

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Data sharing not applicable to this article as no datasets were generated or analysed during the current study

Figure legends

Figure 1. Selection of studies by search strategy. A) Flow diagram of the study selection process. B) Schematic representation of selected articles on glioma cfNAs.

Figure 2. Circulating nucleic acids in blood and CSF. A) Circulating miRNAs and lncRNAs; red: cfRNAs more reliable as biomarkers; B) circulating DNA; red: methylation, blue: deletions, black: mutations.

Figure 3. Circulating non-coding RNA correspond to glioma grade and molecular markers.

Figure S1. QUADAS-2 assessment. Bar diagram showing the risk of bias and applicability concerns of the selected articles, expressed as percentage.

Figure S2. Schematic representation of the most frequently examined glioma categories in cfRNA reports.

Table 1. Studies on circulating RNAs as non-invasive biomarkers in glioma based on our analysis.

Reference	Biomarker	Detection method	Case size	Biofluid	Total or exosomal	Reason of interest
15	miR-21 miR-15b	Real time PCR	10 gliomas vs 40 controls	CSF	total	Diagnostic
20	miR-10b miR-21	Real time PCR	19 GBM vs 74 metastasis and 15 controls	CSF	total	Diagnostic, monitoring
79	mRNA (IDH1)	BEAMing and digital PCR	24 gliomas vs 6 controls	CSF	EV	Detection
24	miR-21	Real time PCR	28 GBM vs 28 controls	CSF	exosomal	Diagnostic
38	miR-451 miR-711 miR-935 miR-223 miR-125b	Real time PCR	13 gliomas vs 14 controls	CSF	total	Diagnostic
55	miR-30e miR-140 let-7b miR-10a miR-21-3p miR-10b miR-196b	Sequencing and real time PCR	175 brain tumours vs 40 controls	CSF	total	Diagnostic, prognostic
43	miR-548c miR-520f miR-27b miR-130b miR-21 miR-218 miR-193b miR-331 miR-374a	Microarray and real time PCR	105 GBM and 90 controls	CSF	exosomal and total	Diagnostic
81	mRNA (EGFRvIII)	Sequencing	71 gliomas	CSF	exosomal	Detection
35	mir-21	Real time PCR	70 gliomas vs 25 controls	CSF and serum	exosomal	Diagnostic, monitoring

80	mRNA (EGFRvIII)	Sequencing	13 gliomas vs 6 controls	blood	EV	Detection
19	miR-21	Real time PCR	25 GBM vs 30 controls	serum	exosomal	Diagnostic
23	miR-15b* miR-23a miR-133a miR-150* miR-197 miR-497 miR-548b-5p	Solexa sequencing and real time PCR	123 gliomas vs 127 controls	serum	total	Diagnostic, monitoring
27	mir-320 miR-574-3p RNUB6-1	Microarray and real time PCR	75 GBM vs 55 controls	serum	exosomal	Diagnostic
44	miR-125b	Real time PCR and meta-analysis	33 gliomas vs 33 controls	serum	total	Diagnostic
28	miR-15b-5p miR-16-5p miR-19a-3p miR-19b-3p miR-20a-5p miR-106a-5p miR-130a-3p miR-181-5p miR-208a-3p	Microarray and real time PCR	150 gliomas vs 160 controls	serum	total	Diagnostic, prognostic, monitoring
29	miR-210	Real time PCR	136 GBM vs 50 controls	serum	total	Diagnostic, prognostic
30	mir-128	Real time PCR	151 gliomas vs 105 controls	serum	total	Diagnostic, monitoring
45	miR-205	Real time PCR	83 gliomas vs 55 controls	serum	total	Diagnostic, prognostic, monitoring
70	miR-210	Real time PCR	91 gliomas vs 50 controls	serum	exosomal	Diagnostic, prognostic, monitoring
42	miR-451a	Real time PCR	118 gliomas vs 84 controls	serum	total	Diagnostic
31	miR-29a miR-29b	Real time PCR	83 gliomas vs 69 controls	serum	total	Diagnostic

	miR-29c					
46	miR-145-5p miR-222-3p miR-182 miR-20a-5p miR-106a-5p	NanoString and real time PCR	106 GBM	serum	total	Prognostic
61	miR-29b	Real time PCR	177 gliomas vs 80 controls	serum	exosomal	Diagnostic, prognostic, monitoring
67	miR-145-5p	Real time PCR	169 gliomas vs 50 controls	serum	total	Diagnostic, prognostic
64	miR-769-3p	Real time PCR	113 gliomas vs 95 controls	serum	total	Diagnostic, monitoring
65	miR-4763-3p miR-1915-3p miR-3679-5p	Microarray	170 gliomas vs 410 controls	serum	total	Diagnostic
66	miR-34a	Real time PCR	82 gliomas vs 42 controls	serum	total	Diagnostic, prognostic
63	miR-214	Real time PCR	100 gliomas vs 100 controls	serum	total	Diagnostic, prognostic, monitoring
62	mir-100	Real time PCR	95 GBM vs 60 controls	serum	total	Diagnostic, prognostic, monitoring
68	miR-26a miR-21	Real time PCR	15 gliomas vs 11 controls	serum	total	Diagnostic, monitoring
47	miR-1303 miR-130a miR-145 miR-335	Real time PCR	9 pediatric gliomas vs 3 controls	serum	total	Diagnostic
48	miR-21 miR-222 miR-124-3p	Real time PCR	100 gliomas vs 41 controls	serum	exosomal	Diagnostic, monitoring
49	miR-203	Real time PCR	100 gliomas vs 30 controls	serum	total	Diagnostic, prognostic
50	miR-451a miR-4298 miR-485-3p	Microarray and real time PCR	36 GBM vs 12 controls	serum	total	Diagnostic, prognostic

51	miR-1825	Real time PCR	57 gliomas vs 57 controls	serum	total	Diagnostic, prognostic
52	miR-376a miR-376b miR-376c	Real time PCR	100 gliomas vs 50 controls	serum	total	Diagnostic, prognostic
39	miR-137	Real time PCR	64 gliomas vs 64 controls	serum	total	Diagnostic, prognostic, monitoring
41	miR-497 miR-125b	Real time PCR	22 gliomas vs 8 meningiomas vs 15 controls	serum	total	Diagnostic
36	miR-15b miR-21	Real time PCR	30 gliomas vs 82 controls	serum	total	Diagnostic
37	miR-10b miR-21	Real time PCR	28 gliomas vs 10 controls	serum	total	Diagnostic, monitoring
56	miR-193b	Real time PCR	122 gliomas vs 68 controls	serum	total	Diagnostic, prognostic, monitoring
57	miR-365	Real time PCR	31 GBM[1]	serum	total	Diagnostic
58	miR-221 miR-222	Real time PCR	20 GBM vs 20 controls	serum	total	Diagnostic, prognostic, monitoring
59	miR-1238	Real time PCR	13 gliomas vs 13 controls	serum	exosomal	Diagnostic, monitoring
76	LINK-A	Real time PCR	52 gliomas vs 38 controls	serum	total	Diagnostic
74	HOTAIR GAS5	Real time PCR	106 GBM	serum	total	Prognostic
77	GASL1	Real time PCR	62 gliomas vs 52 controls	serum	total	Diagnostic, prognostic, monitoring
78	SBF2-AS1	Real time PCR	20 GBM	serum	exosomal	Diagnostic, prognostic
75	HOTAIR	Real time PCR	43 GBM vs 40 controls	serum	exosomal	Diagnostic, prognostic
73	TUSC7	Real time PCR	206 gliomas	serum	total	Prognostic

72	miR210HG	Real time PCR	42 gliomas vs 10 controls	serum	total	Diagnostic
21	miR-21	Real time PCR	10 GBM vs 10 controls	plasma	total	Diagnostic and monitoring
22	miR-21 mir-128 miR-342-3p	Real time PCR	50 gliomas vs 10 controls	plasma	total	Diagnostic and monitoring
32	mir-454-3p	Real time PCR	70 glioma vs 70 controls	plasma	total	Diagnostic, prognostic, monitoring
25	miR-576-5p miR-340 miR-626 mir-320 miR-7-5p let-7g-5p	Microarray	3 GBM and 3 controls	plasma	total	Diagnostic
26	miR-221 miR-222	Real time PCR and meta-analysis	50 gliomas vs 51 controls	plasma	total	Diagnostic, prognostic
34	miR-21 miR-24 miR-125	Real time PCR	9 GBM	CSF and plasma	exosomal	Detection
33	mir-185	Real time PCR	66 gliomas vs 77 controls	plasma	total	Diagnostic, prognostic
69	miR-210 miR-185 miR-449 miR-5194	Real time PCR	50 gliomas vs 15 controls	plasma	exosomal	Diagnostic
71	miR-124	Real time PCR	64 gliomas vs 40 controls	plasma	total	Diagnostic, prognostic
54	miR-182-5p miR-328-3p miR-339-5p miR-340-5p	Sequencing	26 gliomas vs 35 controls	plasma	exosomal	Diagnostic

	miR-485-3p miR-486-5p miR-543 miR-144-5p miR-134-5p miR-493-3p miR-433-3p miR-382-5p miR-379-5p miR-370-3p miR-127-3p miR-381-3p miR-409-3p					
53	miR-122	Real time PCR	74 gliomas vs 74 controls	plasma	total	Diagnostic, prognostic
40	mir-182	Real time PCR	112 gliomas vs 54 controls	plasma	total	Diagnostic, prognostic
60	miR-449a	Real time PCR	30 gliomas vs 12 controls	plasma	total	Diagnostic

[1]No information about controls

Table 2. Studies on circulating DNA as non-invasive biomarker in glioma based on our analysis.

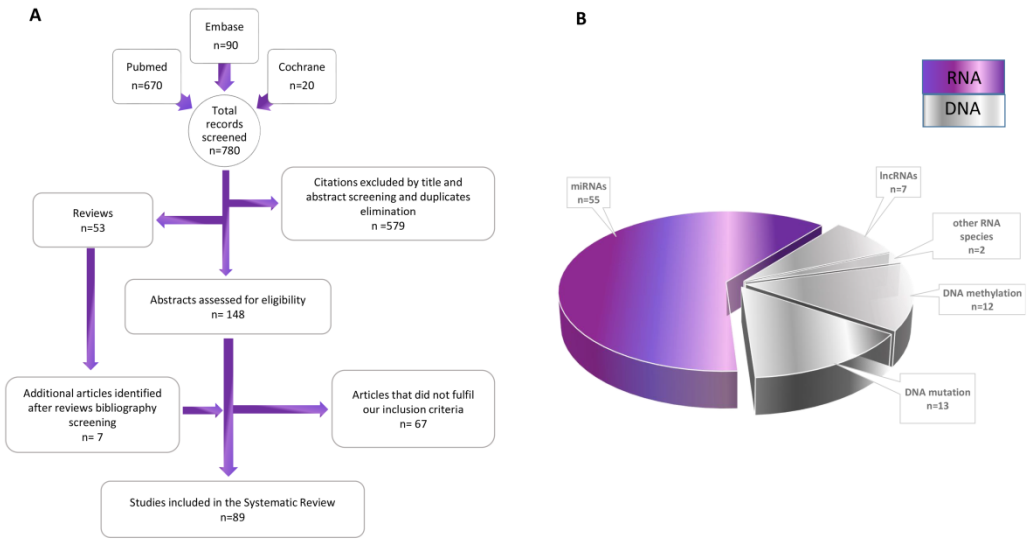
Reference	Biomarker	Detection method	Case size	Biofluid	Total or exosomal	Reason of Interest
95	A panel of cancer-associated genes (most relevant *TERT promoter, TP53, IDH1, NF2, PIK3R1, PTCH1, PTEN)*	Sequencing	29 gliomas vs 6 medulloblastomas	CSF	total	Diagnostic
97	341 cancer-associated genes (most relevant *CD4K AMP, IDH1R132H, 1p/19q del, PDGFRA AMP, CDKN2B, EML4-ALK* gene fusion*)*	MSK-IMPACT	12 gliomas	CSF	total	Diagnostic
104	410 cancer-associated genes (most relevant *EGFR, CDKN2A/CDKN2B, EGFR*-variant III, 1p/19q codeletion, *TERT* promoter, *TP53, IDH1)*	Sequencing	85 gliomas	CFS	total	Diagnostic, monitoring
103	*IDH1, IDH2, TP53, TERT, ATRX, H3F3A, HIST1H3B*	Sequencing	20 gliomas	CFS	total	Diagnostic
101	Mutation, copy number alteration (most relevant *IDH1, IDH2, TP53, CDKN2A/CDKN2B, EGFR)*	Whole genome sequencing	13 gliomas	CSF	total	Diagnostic
82	*MGMT, P16, DAPK, RASSF1A*	MSP	28 GBM	serum	total	Monitoring

85	*MGMT, PTEN, 10q, 1p/19q*	MSP and real time PCR	70 gliomas vs 20 controls	serum	total	Prognostic
84	*p16*	MSP	40 gliomas	serum	total	Diagnostic
99	*ALU, MGMT, P16, RASSF1A*	MSP	124 gliomas vs 58 controls	serum	total	Diagnostic, Prognostic
90	*ALU*	BSP	65 gliomas vs 30 controls	serum	total	Diagnostic, Prognostic
89	*RASSF1A, p15INK4B, MGMT, p14ARF*	MSP	17 gliomas	serum	total	Diagnostic
83	*p16/(INK4a), MGMT, P73, RARbeta*	MSP	10 gliomas	plasma	total	Monitoring
87	*IDH1*	COLD PCR and digital PCR	80 gliomas vs 31 controls	plasma	total	Diagnostic
92	*MGMT* methylation	Real time PCR	58 gliomas	plasma	total	Monitoring
91	*EGFRvIII*	Long range PCR amplification	13 GBM	plasma	total	Monitoring
105	Panels of cancer- associated gene *(most relevant IDH1, TERT, CDKN2A/CDKN2B, EGFR, P53, PTEN, NTRK* fusion*)*	NGS	419 primary brain tumor	plasma	total	Monitoring
96	*ALU*	BSP	109 gliomas	serum	total	Diagnostic, Prognostic
106	Panel of cancer- associated gene *(most relevant IDH1*, MGMT*, TP53, EGFRvIII, , NF1, PTPN11, PDGFRA, PIK3CA, STAG2)*	NGS	42 gliomas	plasma	total	Prognostic

86	*MGMT, p16INK4a, TIMP-3, THBS1*	MeDIP (Methylated DNA Immunoprecipitation) and real time PCR	86 gliomas	CSF and serum	total	Prognostic, monitoring
88	*ALU*	Real time PCR	70 gliomas vs 20 controls	CSF and serum	total	Diagnostic
93	*MGMT*	MSP	89 gliomas	CSF and serum	total	Diagnostic, Prognostic
94	*PTEN, IDH1*	Digital PCR	12 gliomas	CSF and plasma	total	Diagnostic
98	*H3K27M*	Digital PCR	48 gliomas vs 36 controls	CSF and serum	total	Monitoring
102	Alteration in DNA	Sequencing	200 Patients 65 controls	plasma	total	Diagnostic
100	Promoter TERT mutation	Real time PCR and Ion Torrent PGM NGS system	60 gliomas	CSF and plasma	total	Prognostic
17	DNA-methylation profiles	cfMeDIP-seq	220 Patients	plasma	total	Diagnostic

MSP: Methylation-specific PCR

BPS: Bisulfite sequencing PCR



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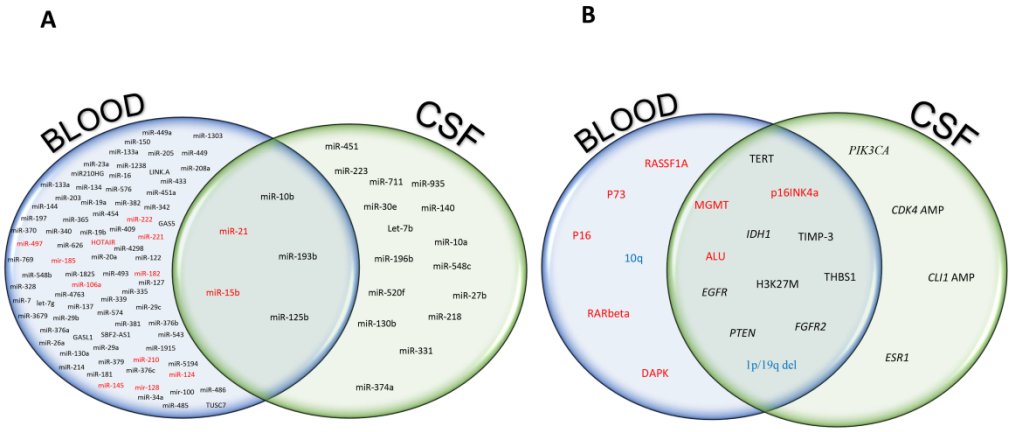

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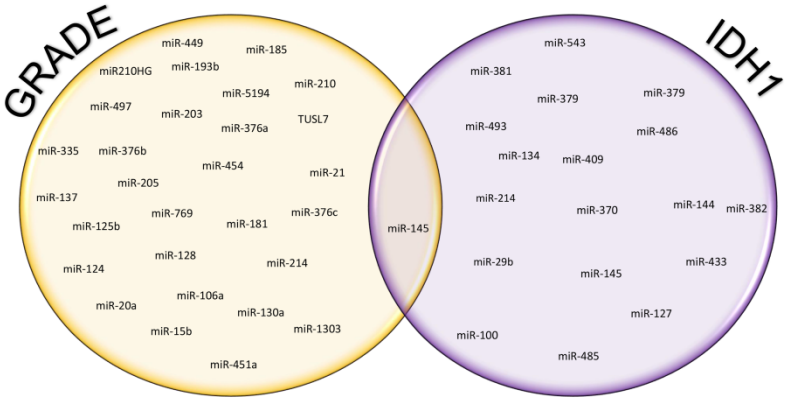

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