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Metabolomic signature of brain cancer

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Abstract

Despite advances in surgery and adjuvant therapy, brain tumours represent one of the leading causes of cancer-related mortality and morbidity in both adults and children. Gliomas constitute about 60% of all cerebral tumours, showing varying degrees of malignancy. They are difficult to treat due to dismal prognosis and limited therapeutics. Metabolomics is the untargeted and targeted analyses of endogenous and exogenous small molecules, which characterizes the phenotype of an individual. This emerging “omics” science provides functional readouts of cellular activity that contribute greatly to the understanding of cancer biology including brain tumour biology. Metabolites are highly informative as a direct signature of biochemical activity; therefore, metabolite profiling has become a promising approach for clinical diagnostics and prognostics. The metabolic alterations are well-recognized as one of the key hallmarks in monitoring disease progression, therapy and revealing new molecular targets for effective therapeutic intervention. Taking advantage of the latest high-throughput analytical technologies, *i.e.* nuclear magnetic resonance spectroscopy and mass spectrometry, metabolomics is now a promising field for precision medicine and drug discovery. In the present report, we review the application of metabolomics and *in vivo* metabolic profiling in the context of adult gliomas and paediatric brain tumours. Analytical platforms such as high-resolution nuclear magnetic resonance, *in vivo* magnetic resonance spectroscopic imaging and high- and low-resolution mass spectrometry are discussed. Moreover, the relevance of metabolic studies in the development of new therapeutic strategies for treatment of gliomas are reviewed.

Keywords

adult glioblastoma; paediatric glioblastoma; metabolomics; mass spectrometry; magnetic resonance spectroscopy

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Introduction

Although there are numerous brain tumour types, gliomas are the most common primary brain tumours of the central nervous system (CNS) in adults and originate from glial cells, such as astrocytes, oligodendrocytes and ependymal cells [1]. Based on the histopathological and clinical criteria, gliomas are classified into grade I to IV by the World Health Organization (WHO) [2, 3]. Grade IV astrocytoma, or glioblastoma (GBM), is the most aggressive type of brain malignancy. Despite numerous treatment strategies, little therapeutic progress has been achieved over the last few decades [3, 4]. Survival rates of 27% and 10% were reported at 2 and 5 years from diagnosis [5]. Median progression-free survival and median overall survival from diagnosis of 6.2–7.5 months and 14.6–16.7 months, respectively were reported in GBM clinical trials [5]. Novel strategies are urgently needed for development of new therapeutic approaches [6]. Metabolomics, the youngest sibling in the systems biology family, can not only complement genomics, transcriptomics and proteomics, but also add a novel dynamic prospective to precision medicine and drug discovery [6, 7].

There are numerous challenges to treating GBM tumours. The blood brain barrier and poor tumour vasculature restrict many potential drugs from reaching these tumour cells. Furthermore, these tumours are characterized by highly disordered vascular proliferation and pseudopalisading necrosis, in which necrotic hypoxic foci are surrounded by dense migrating tumour cells escaping hypoxia. A pattern of disordered vascular growth, necrosis, hypoxia, and cell migration contributes to the infiltrative nature and poor prognosis of these tumours [8]. Given the prevalence of hypoxia and migratory nature of tumour cells, angiogenesis is necessary for these cells to receive adequate access to oxygen and nutrients. It is not surprising that GBM has been classified as one of the most endothelial-dependent tumour types [9]. For this reason, the VEGF inhibitor bevacizumab has been implemented as an anti-angiogenic agent in the treatment of refractory tumours. Unfortunately, although bevacizumab extends progression-free survival, it fails to extend overall survival in GBM patients, indicating that initially sensitive tumours develop resistance to anti-angiogenic therapy over time [10]. Tumour hypoxia has been quantified in patients treated with bevacizumab, and high tumour hypoxia (measured by hypoxia marker CA9) has been linked to increased resistance to treatment and poor patient prognosis, even though many highly hypoxic tumours initially show radiographic response to bevacizumab [11]. Hypoxic resistance is not unique to anti-angiogenic therapy, and is a feature of radiation therapy and a variety of chemotherapies in numerous cancer types [12].

It is likely that long-term metabolic reprogramming is involved in the development of this treatment resistance. Recurrent tumour growth under hypoxic conditions would require proliferating cells to be less reliant on the citric acid cycle and oxidative phosphorylation for energy production and more reliant on glycolysis and lactic acid production. Malignant cells are typically more reliant on glycolysis than oxidative phosphorylation for their energy needs even in normoxia, a phenomenon known as the Warburg effect, but this metabolic behaviour is further exacerbated under hypoxia [12]. This metabolic feature can be beneficial for cells subjected to radiation, by decreasing their oxidative stress load. Another possible explanation for hypoxic resistance to therapy is that metabolic adaptations under

hypoxia channel metabolites into alternative pathways that may confer long-term advantages onto chronically hypoxic tumours. These alternatively elevated pathways can lead to the synthesis of structural or signalling metabolites including lipids, amino acids, and nucleic acids, and other metabolites necessary for cellular proliferation, tumour growth, and tumour cell survival. Metabolic adaptations in GBM may also be playing an important role in evasion of the immune system by tumour cells, as well as maintenance of tumour stem cells, which are highly resistant to stresses induced by chemotherapy and radiation [13–15]. Metabolomic analysis will help determine the important pathways that can be targeted in order to combat treatment resistance in glioma. Beyond adult gliomas, metabolic patterns are useful in differentiating and determining the prognosis of different paediatric brain tumour types [201–212]. Further work needs to be done to translate metabolic findings into potential treatment targets.

Metabolomics refers to the untargeted and targeted analyses of the metabolome, *i.e.* the complete set of all the endogenous and the exogenous metabolites (small molecules <3000 Da) present in a biological system such as cells, tissues and biofluids (e.g. cerebrospinal fluid(CSF), plasma, urine and saliva) [16, 17]. It provides a direct readout of physiochemical reactions involving genome, transcriptome, proteome as well as environmental influences within the cell, the tissue or the entire organism that include dietary pattern, life style, disease status and gut microbiota [17, 18]. The functions of genes and proteins undergo epigenetic regulations and post-translational modifications respectively, whereas metabolites act as a direct indicator of biochemical or enzymatic activity, and correlate with the biochemical changes within the phenotype of an individual [18, 19]. Therefore, deciphering the metabolome is a sensitive and robust approach for monitoring changes in a biological system and identifying pathways that are perturbed in a given pathology through observed changes in the metabolic network. A comprehensive and holistic understanding of the metabolic differences between cancer and normal cells or between different cancer subtypes might provide insight into novel therapeutic targets for untreatable gliomas. Moreover, metabolomics offers several practical advantages over transcriptional and proteomic approaches, due to the characteristic high-throughput, fully automated and relatively low cost methods [17, 18]. Recent advancement in high-throughput metabolomics technologies, *i.e* nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) analyses, has improved the sensitivity and resolution of analytic assays to achieve a comprehensive biochemical assessment. Capitalizing on the latest generation of high-resolution analytical platforms, hundreds to thousands of small molecules can be identified and quantified in an untargeted or unbiased fashion from a small biological sample size. In this context, metabolomics has become a promising strategy that has been widely adopted for tumour diagnosis, monitoring tumour growth and regression, pathogenic mechanisms as well as monitoring response to treatment regimens [17, 18, 20–23]. Metabolomic investigations have already proved their importance in recognizing numerous disease-associated characteristic metabolic differences in cancer versus normal cells in colon, leukemia, ovarian, oesophageal, oral, breast and prostate cancers [24–33]. These investigations have also been extended to brain cancer studies and have provided novel biomolecular insights into the aggressive phenotype of this malignancy [34, 35]$^{-}$. In this review, metabolomics

strategies/methods and their major applications in adult and paediatric brain tumour diagnostics and treatments are discussed.

1. Metabolic profile analysis methods

Methods to analyse small metabolites in biological samples can be essentially divided into three methodological approaches: untargeted metabolomics, metabolic profiling, also referred to as targeted metabolomics, and stable isotope-resolved metabolomics (SIRM) [36–39]. Metabolic profiling is commonly used to identify and quantify a predefined list of metabolites from a biological sample [40]. On the other hand, untargeted metabolomics refers to an unbiased, hypothesis-free analysis of all detectable metabolites from a biological sample [19, 36]. Despite the huge amount of features detected using an untargeted metabolomics analysis, often this approach represents a compromise between specificity and selectivity, and time of acquisition. In fact, a comprehensive analysis cannot be achieved by using one single analytical technology or by performing the metabolic analysis in a single acquisition. However, an untargeted approach is advantageous in pinpointing metabolites that are highly affected by the system perturbation (for instance, disease or pharmacological intervention). Once the metabolic pathway of interest is identified using an untargeted approach, a targeted metabolic profiling may sometimes be required to increase the number of metabolites associated with that specific pathway [19, 37]. In spite of all the technological advances of the analytical methods, the identification and quantification of several metabolites can be trivial and requires continuing development of appropriate protocols. A more in depth discussion of some of these metabolites will be reviewed later in the “Detection of critical metabolites” section.

In addition to untargeted and targeted metabolomics, SIRM is a robust metabolic approach for dynamically tracing the fate of selected isotopically labelled compounds and unequivocally assign the metabolic alteration to a specific pathway. This approach requires *a priori* selection of the tracers that need to be replaced in culture media (*e.g.* glucose, glutamine) or injected in a living organism before sample collection [41, 42]. Because many metabolites can be found in several cellular compartments, the use of the appropriate tracers might provide relevant insights into metabolites that have been labelled exclusively in a specific cell compartment [43–46]. SIRM typically uses carbon (^{13}C), nitrogen (^{15}N) deuterium (^{2}H), and oxygen (^{18}O) metabolic datasets generated using stable isotope-assisted metabolomics to calculate intracellular and extracellular metabolic fluxes. The stable isotopes most widely utilized in brain cancer studies include ^{13}C-glucose, ^{13}C-glutamine, and ^{13}C-acetate [47–49].

A wide array of analytical techniques suitable for the detection of small metabolites are available, including ultraviolet-visible spectroscopy (UV), Fourier transform infrared spectroscopy (FT-IR), and Raman spectroscopy; however, the most widely utilized analytical techniques in cancer metabolomics are MS and NMR spectroscopy [18, 19, 50].

2.1. NMR spectroscopy

NMR spectroscopy, a quantitative high-throughput metabolomics technology, constitutes a useful tool in metabolomics research, yielding both qualitative and quantitative information

[51]. It is a rapid, non-destructive, non-selective and highly reproducible technique that requires minimal sample preparation and provides highly informative structural information [52, 53]. NMR techniques detect different ½ spin nuclei, including ^{1}H, ^{31}P, ^{13}C, and ^{15}N, in metabolites based on their resonance frequency when placed in a magnetic field. Mostly ^{1}H and ^{13}C NMR spectroscopy are used for NMR-based metabolomics; in addition, ^{31}P NMR spectroscopy is specifically used to measure high-energy phosphate metabolites (e.g. ATP, creatine phosphate), and phosphorylated metabolic and lipid intermediates [18, 54–56].

For SIRM applications, NMR spectroscopy has been historically used for structural elucidation of metabolic tracers by determining the site-specific incorporation by *in vivo* and *in vitro* ^{13}C NMR [57–60]. Alternatively, two dimensional NMR spectroscopy [61, 62] acquired using a large number of increments on the indirect dimension can provide ^{13}C-^{13}C scalar coupling information and thus valuable information to determine the exact position of the labelled atom [63, 64]. ^{13}C NMR has been successfully applied to study tumour metabolism of primary human GBM and IDH-mutated gliomas by infusion of ^{13}C-labeled nutrients (glucose, acetate and glutamine) [47–49].

Magnetic resonance spectroscopic imaging (MRSI) is a non-invasive technique that combines the chemical specificity of NMR spectroscopy with the spatial localization capabilities of MRI and is well-suited for *in vivo* studies of metabolic changes in brain disorders such as brain tumours, seizure disorders and Alzheimer's disease, without exposure to ionizing radiation [65–67]. It is used to determine the relative concentrations of target brain metabolites; however, a limited number of metabolites can be identified by MRS due to its low sensitivity and spectral resolution. High-resolution magic angle spinning (HR-MAS) ^{1}H NMR spectroscopy can profile intact tissue, allowing the simultaneous analysis of aqueous and lipid-soluble metabolites, and provides information about the metabolic tumour microenvironment [18, 55]. It generates high-resolution spectra and requires little or no sample preparation. Recent development in NMR technology such as introduction of stronger magnetic fields, microcoil probes, cryogenic probes, advanced pulse sequences, and isotope labelling, have significantly enhanced NMR performance [53]. Despite these advantages, NMR spectroscopy is not suitable for detection of metabolites present at trace levels owing to its low sensitivity and poor dynamic range, therefore mass spectrometry is often combined to NMR for a more comprehensive metabolomics analysis [68, 69].

2.2. Mass spectrometry

Mass spectrometry (MS) provides a valuable analytical platform for metabolomics due to its high sensitivity and wide dynamic range [70]. It is a versatile technique owing to the myriad of instruments differing in operational principles and performance [52, 70]. A variety of mass analysers with different ion acceleration and detection methods are available and include high-resolution (HR; *e.g.* time of flight (TOF), Fourier transform ion cyclotron resonance (FT-ICR), orbital detector (Orbitrap)) and low-resolution (LR; *e.g.* single quadrupole (Q), triple quadrupole (QqQ) and ion trap (IT)) [52, 70, 71]. Also, there are different interfaces for the generation of ions: hard ionization techniques, such as electron impact (EI), and soft ionization techniques, including chemical ionization (CI), electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), atmospheric pressure

photoionization (APPI), matrix-assisted laser desorption ionization (MALDI), and desorption electrospray ionization (DESI) [52, 70, 71]. Each of these MS techniques has its own pros and cons and they have been extensively discussed elsewhere [52, 71].

Recently, tandem mass spectrometry (MS/MS) or MS^n analysis enabled combination of same or different detectors, hybrid mass analyzers for structural elucidation and quantitative analysis of metabolites [71–74]. The fragmentation pattern of metabolites in MS^n analysis is mainly achieved by low energy dissociation such as collision induced dissociation (CID) and high energy collision dissociation (HCD); integration of both CID and HCD using hybrid IT-Orbitrap, allows to eliminate identification of ambiguous metabolites [75, 76]. The HR tandem mass spectrometers such as IT-Orbitrap, Q-TOF, and IT-TOF provide accurate precursor and product ion information, isotopic distribution, and elemental composition of compounds with high mass accuracy, which significantly enhances the metabolite characterization reliability [77, 78]. Hence, these methods are preferable for untargeted metabolomics [19, 78]. On the contrary, LR tandem mass spectrometers such as triple quadrupole (QqQ) and hybrid linear ion trap triple quadrupole (QqQ_{LIT}) using single reaction monitoring (SRM) and multiple reaction monitoring (MRM) acquisition ensure good selectivity and sensitivity for quantitative analysis or metabolic profiling [19, 77, 79].

In metabolomics applications, mass spectrometers are typically hyphenated to either gas chromatography (GC-MS) or liquid chromatography (LC-MS), to facilitate separation of complex biological mixtures before MS detection [52, 70]. GC-MS, typically using either EI or CI, is ideally suited for the analysis of volatile and thermally stable metabolites [52, 70]. GC-MS is also used to analyse semi-volatile or non-volatile metabolites through chemical derivatization; however this leads to long analysis time and sample preparation variability[70]. In addition, the derivatization artefacts, such as multiple MS peaks per analyte and multiple analytes per peak due to incomplete derivatization, degradation and side reactions, make spectral interpretation and identification more challenging [80]. Lately, the development of GC-QLT-Orbitrap hybrid instrument makes significant improvement in the GC/MS-based metabolomics for trace analysis and structural characterization of unknown metabolites [81]. It facilitates high mass accuracy, high resolution, high sensitivity and fast scan speed with the unambiguous determination of elemental compositions and isotopic distribution of metabolites [81].

In contrast to GC-MS, LC-MS combined with API (ESI, APCI and APPI) interface provides excellent combination of sensitivity (up to picomole to femtomole levels), selectivity and specificity for the analysis of non-volatile and thermally labile metabolites having wide range of polarity with little effort in sample preparation [36, 82]. Mostly, ESI is utilized in LC-MS based metabolomics due to its applicability to a wide range of analytes (highly to moderately polar), amenability to automation, good quantitative capacity and reproducibility [71].

Mass spectrometric measurements (either GC-MS or LC-MS) are widely employed for the detection of stable isotopes (*e.g.* SIRM) because of their sensitivity in identifying a large number of isotopomers by mass [44, 46]. However, isotopomers identified only by mass (m +1, m+2, m+3…) do not provide positional isotopic labelling information. For example, the

5-carbon compound glutamate can be present in 6 different isomers (m, m+1, m+2, m+3, m +4, m+5) with 32 different positional isotopomers. For a detailed analysis of the positional isotopic enrichments, NMR or tandem mass spectrometry need to be employed [83, 84]. The latest developments in tandem mass spectrometry (MS/MS) allows determination of positional labelling based on mass fragmentation for isotopically-labelled ^{13}C amino acids [85, 86] and other intermediates of glycolysis, pentose phosphate pathway and TCA cycle intermediates [87].

2.3. Detection of critical metabolites

The metabolomics community is constantly engaged in developing and optimizing new protocols to improve confidence in identification and quantification of metabolic biomarkers. The major challenges in metabolomics analysis are intrinsic to the diverse chemical and physical properties of each metabolite. Very fast turnover of metabolism and limited half-life of many metabolites, as they are constantly undergoing oxidation/reduction or degradation during biochemical reactions, are omnipresent challenges [36, 37]. Therefore, it is important to carefully design an experimental procedure, including sample collection, storage and processing, chromatographic separation, analytical instrumentation, and data processing and analysis that maximizes metabolite detection and quantitative reproducibility, ultimately yielding high-quality data [19, 88]. For instance, sample collection and sample preparation need to be optimized based on the biological sample [89]. Most commonly used sample-preparation methods for biofluids are “dilute-and-shoot”, solvent precipitation and ultrafiltration [89]. Among these methods, ultrafiltration provides improved removal of protein, excellent coverage of polar metabolites and direct extraction of non-polar metabolites from membrane using methanol-water followed by chloroform [89, 90]. For cell metabolomics, sampling needs to be adjusted depending on the cell type, either grow as adherent cultures or in suspension [91]. Basic steps of sampling involves rinsing (by trypsinization/ice cold PBS, 60% MeOH and water), metabolism quenching (by liquid nitrogen or ice-cold solvents), cell lysis and metabolite extraction [89, 91–93]. Similarly, tissue sampling involves rinsing to remove blood, quenching, tissue homogenization at low temperature and metabolite extraction [89]. Among different metabolite extraction procedure, liquid–liquid extraction (LLE) is the most commonly used method [91, 93]. Other methods such as solid-phase extraction (SPE) or solid-phase micro-extraction (SPME) are specifically employed for targeted analysis [91, 93]. However, most of these new protocols are often uniquely developed for a specific analytical platform. When the concentration of a metabolite is in the nano- to micro molar range, NMR continues to be a very resourceful platform, particularly due its non-destructive nature, limited susceptibility to sample matrix effects and capability to tolerate buffered solutions (*e.g.* 100 mM phosphate buffer or more) which reduces sample variability. NMR is widely used for absolute quantification [51] and for monitoring the metabolic perturbation of several challenging polar metabolites including those with molecular weight smaller than 50 g/mol (e.g. formate, ethanol, methanol), unstable metabolites (e.g. containing thiol groups, glutathione) or nucleotides (e.g. AMP, ADP and ATP) and cofactors (e.g. NAD and NADH). When the amount of biological sample available for the analysis is very limited or the low concentration of metabolites becomes a limiting factor, high-resolution mass spectrometry is the most resourceful technology currently available, although several critical issues need to

be taken into account and new protocols are constantly being developed to improve accurate identification and quantification of metabolic biomarkers. Examples of challenging metabolites are reported below.

Cysteine metabolism—Metabolites participating in cysteine metabolism such as cysteine, reduced glutathione (GSH) and homocysteine and their corresponding disulfides [cystine, oxidised glutathione (GSSG), and homocystine] are very challenging to analyse as these are highly polar and prone to undergo oxidation/reduction during biochemical reactions [94–96]. Thiol groups (-SH) are labile and easily transformed into corresponding disulfides by oxidative coupling reactions. Therefore, derivatization of the thiol group (-SH) is necessary to accurately analyse these metabolites in biological samples. The pH value of media/mobile phase in thiol analysis is the most crucial parameter and should be maintained below 7, as these compounds rapidly undergo non-enzymatic autoxidation at pH >7 [97, 98]. A variety of analytical methods have been developed for analysis including HPLC, GC and CE coupled with UV and fluorescence detection [94–96, 99–106]. These methods require pre- or post-column derivatization and involve a less sensitive, complicated and time-consuming procedure.

HPLC coupled with electrochemical detector (ECD) allows the analysis of these metabolites in a short time, without derivatization, but suffers from the loss of signal sensitivity in repeated analysis [107]. However, due to high sensitivity and high throughput capability, LC-MS/MS method has been widely employed, which involves derivatization of -SH group of samples with thiol-reactive groups such as iodoacetic acid, maleimides, Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid or DTNB), 4-fluoro-7-sulfamoylbenzofurazan (ABD-F), and monobromobimane [108–119]. The derivatization step only protects thiols from autoxidation and degradation, but it does not elude the enzymatic reduction of disulphides. For example, Seiwert and colleagues used ferrocene-based maleimides in a LC-MS/MS application for derivatization of both disulfides and thiols [120]. Recently, a few LC-MS/MS studies have reported direct and simultaneous quantitation of oxidized and reduced forms of GSH in cells and physiological fluids [98, 121–125]. In direct LC-MS/MS analysis, ethylenediaminetetraacetic acid (EDTA) is used for treatment of samples stored at −80°C and trichloroacetic acid for deproteinization which minimizes thiol oxidation [98, 125].

Nucleotides—Nucleotides play important role in several biochemical pathways, including DNA synthesis and mitochondrial oxidative phosphorylation, and full characterization of these metabolites is potentially critical in particular for quantification of redox active (NAD/NADH, NADP/NADPH, and FAD/FADH) and hydrolytically unstable nucleotides(e.g. ATP/ADP/AMP). Various research groups developed reliable methodological approaches, including MS coupled with effective chromatographic techniques such as capillary electrophoresis CE, hydrophilic interaction chromatography (HILIC), and ion-pairing chromatography (IP-LC) for the analysis of critical nucleotides [126–139]. However, the latter two are the more recently developed and most commonly used techniques, despite IP-LC still providing greater reproducibility, better peak shape, and broad range. Ion paring reagents (IPRs) improve retention time of polar compounds but have drawbacks such as reduced sensitivity, ion suppression, MS source contamination, and adduct formation. The

pH of mobile phases, use of different columns, and IPRs are crucial factors for analysis. Several IPRs such as diamyl ammonium (DAA) acetate, dimethylhexylamine (DMHA), hexylamine, tributylamine, and organic di-/poly cations have been used for nucleotide detection in positive and negative ESI mode [126–139]. The volatile IPRs, DAA acetate, and DMHA were found to be more effective in that they significantly reduce the ion suppression in ESI, increase sensitivity, and avoid MS source contamination with regular cleaning [134, 135]. Furthermore, hypercarb porous graphitic carbon (PGC) and mixed-mode stationary phase have also been applied for nucleotide analyses, as they limits the use of IPRs [140–142].

2-hydroxyglutarate—D-2-hydroxyglutaric acid (D-2-HG) is an important oncometabolite and well-recognized diagnostic marker of IDH mutations in several cancers including low grade gliomas [143–148]. Identification and quantification of 2-hydroxyglutaric acid (2-HG) is not challenging, but characterization of the *D* and *L* enantiomer fractions is still critical and requires extensive sample preparation. The use of chiral columns for HPLC analysis is not fully developed and requires a significant amount of material, therefore, this technology is rarely used in cancer research and is only feasible for enantiomeric analysis of abundant biological samples (*e.g.* urine) in metabolic disorder studies [149, 150]. To differentiate *D* and *L* forms of 2-HG, 2-HG enantiomers are converted into diastereoisomers by derivatizing chiral carbon [146]. Analytical determinations of 2-HG enantiomers by GC-MS and LC-MS have been achieved by using two strategies: utilizing chiral stationary phase [150, 151] and chiral derivatization [145, 146, 152–154]. Several chiral derivatization reagents, *e.g.* acetic anhydride, (D)-2-butanol, ethyl chloroformate, O,O'-diacetyl-L-tartaric anhydride (DATAN), and N-(p-toluenesulfonyl)-L-phenylalanyl chloride (TSPC), have been used in previous reports[145, 146, 152–154]. O,O'-diacetyl-L-tartaric anhydride (DATAN) and N-(p-toluenesulfonyl)-L-phenylalanyl chloride (TSPC)are widely utilized reagents and TSPC offers much better detection sensitivity and derivatization selectivity [145, 146, 153].

In contrast to LC-MS, reported GC-MS methods require two-step derivatization processes and offer poor separation of enantiomers [152, 154]. Moreover, LC-MS methods were used to analyse 2-HG enantiomeric forms in MRM mode for more specific analysis [145, 146, 153]. So far, only for a few studies have reported detection of *D*-2-HG and *L*-2-HG using chiral stationary phase [150, 151]. Das Neves *et al.* used chiral column (fused silica capillaries coated with 1(R)-trans-N,N'-l,2-cyclohexenylbisbenzamideoligosiloxane) in GC-MS with single ion monitoring (SIM) [151] and Rashed et al. used ristocetin A glycopeptide antibiotic silica gel bonded chiral column combined with mass spectrometry in MRM mode to detect D-2HG and L-2HG [150].

One-carbon metabolism—Formate plays a central role in multiple biochemical pathways including one carbon metabolism and *de novo* purine biosynthesis, and its analysis is challenging due to its small molecular weight (46 g/mol) [155]. NMR is routinely used to measure formate [156]. In addition, GC coupled with FID, ECD and MS have been reported and involve esterification of formic acid prior to analysis using a variety of derivatizing reagents [157–163]. Selection of a suitable derivatizing reagent, optimum concentration of reagents and catalysts, and pH of buffer solution are the key concerns in analytical

development due to instability and easily hydrolysable nature of formate esters [157, 158]. Pentafluorobenzyl bromide (PFBBr) has been reported to be the most suitable derivatizing reagent owing to its better retention on the GC column, higher sensitivity, and improved stability of formate ester at pH 6.8–8.0 [158, 162–165]. Some GC-FID and GC-MS studies have been performed using headspace solid-phase microextraction (HS-SPME) and HS in-tube extraction (ITEX) to increase detection sensitivity of formate in biological fluids [160, 161]. Furthermore, for more accurate and sensitive analysis isotope-dilution GC-MS assays have been reported using ^{13}C-formate [157, 162, 163].

Based on the relevance of these key intermediate targets of several chemotherapeutic treatments, optimized and more high-throughput protocols will be developed in the near future.

3. Metabolomics studies in adult brain tumours

Altered cell metabolism is one of the hallmarks of cancer and plays a key role in promoting tumorigenesis, sustaining tumour growth, and improving tumour resistance to chemotherapy [166–168]. Metabolic alteration of glucose, fatty acid, and anaplerotic amino acid metabolism has been associated with malignant transformation and drug resistance in brain cancer [169]. Recently, metabolomics studies indicated that ‘metabolic phenotypes’ possess great potential for development of novel therapeutics and monitoring of treatment response in brain tumours[34, 35]. Therefore, a relevant number of studies focus on the metabolic characterization of immortalized and primary isolates or cultured cells which include neurones, glial cells, astrocytes and neural stem cells and their benign and malignant counterparts [170–173]. Updated lists of the metabolic alterations in adult and paediatric brain tumours associated with neuroblastoma, glioblastoma (low and high grade gliomas), meningioma, and other rare malignant brain tumours are reported in Tables 1 and 2. Both NMR and MS technologies have established and are currently used to expand the number of metabolic biomarkers associated with tumour stratification and predictive of outcome to specific brain cancer treatments [34, 35]. For example, the elevation of hexosamine pathway metabolic intermediates in brain cancer cell lines (rat GBM, human GBM, human supratentorial primitive neuroectodermal tumour (ST-PNET) and human medulloblastoma)are linked to cancer cell death following cisplatin treatment [172]. NMR analysis of glioblastoma stem cells identified a high level of α-aminoadipate (αAAD) in GBM as a marker of tumour aggressiveness [174].

Despite the limited number of primary samples compared to the *in vitro* experiments, several labs have analysed brain biopsies, plasma/serum, urine and CSF samples from astrocytoma and glioblastoma patients. Most of these studies were able to identify distinct metabolic patterns associated with diagnosis and prognosis of brain cancer patients (Table 1) [55, 175–182]. For instance, D-2-HG is a well-established metabolic biomarker for tumour grade classification and chemotherapeutic response in low-grade gliomas (LGGs) [143, 144, 183, 184]. Mutations in the metabolic enzyme isocitrate dehydrogenase 1 (IDH1) have been identified in LGGs and in secondary glioblastoma, and result in a NADPH-dependent reduction of α-ketoglutarate to D-2-HG [143, 144, 183]. IDH mutations associated with an elevated level of D-2-HG contribute to tumour formation and malignant progression in

gliomas, however, a high level of D-2-HG is also a predictor of longer survival and good response to temozolomide (TMZ) in LGGs [143, 144, 185].

Another relevant metabolic biomarker associated with glioma grade is myo-inositol (MI), an important osmolyte and substrate for the synthesis of the phosphatidylinositol lipid family [186, 187]. The increased concentration of MI has been reported in many brain disorders that involve astrocyte proliferation. Kallenberg and colleagues reported that MI level was significantly higher in patients with GBM compared to patients with LGGs and control subjects [187]. Several *in vivo* studies carried out in patients with different grades of astrocytomas have also demonstrated that astrocytomas show low NAA peak and prominent signals from Cho group-containing compounds, and Lac [188–191]. Malignant tumour cells convert most of glucose into lactic acid by active glycolysis, which increases the cytosolic $NADH/NAD^+$ ratio and thereby accelerates lactate dehydrogenase LDH A activity [182]. In GBM, up-regulation of M2 isoform of pyruvate kinase (PK) PKM2 favours aerobic glycolysis, thereby increasing Lac production [192]. A switch to aerobic glycolysis may benefit these tumours during the development of tumour hypoxia by lowering the levels of oxygen consumption needed to sustain energy production. The higher levels of triose phosphate glycolytic intermediates, including phosphoenolpyruvate (PEP) and 3-phosphoglycerate (3-PG), and Lac represent unique markers for gliomas [182, 183].

Recently, Mörén and colleagues performed metabolic profiling on tumour tissue and serum samples from glioma patients and were able to identify a metabolic signature of GBM compared to oligodendrogliomas ([193], Table 1). Turnover of several metabolites have been reported in this study including cysteine (Cys) which has been found upregulated in serum samples of GBM compared to oligodendrogliomas patients [193]. Cysteine metabolism in brain tumour is gaining considerable interest due to its role in GSH synthesis via the cysteine/glutamate antiporter system x_c^- [194–197]. GSH, a tripeptide of Glu, Cys and Gly, is a major cellular antioxidant and key regulator of the redox status of cells in the CNS [195, 197]. GSH synthesis plays a significant role in glioma cell survival during redox stress and hypoxia [197, 198]. Investigation on GSH relation to clinical stage of brain tumours showed significantly lower GSH levels in high grade gliomas (HGGs, III and IV) compared to low grade gliomas (LLGs, I and II) [199, 200]. More recently, a novel metabolic pathway has been identified inducing the generation of cysteine sulfinic acid (CSA), a metabolic intermediate of cystine catabolism, and a23 fold increased accumulation of CSA has been demonstrated in GBM compared with grade II glioma [194]. Therefore, systemic depletion of Cys with cyst(e)inase enzyme [201] for sustained depletion of the extracellular L-Cys and CSSC pool is an attractive strategy to overcome tumour resistance, increasing sensitivity to cancer therapy.

In addition to cysteine, other amino acids are of particular interest for brain cancer. For instance, Glu is the main excitatory neurotransmitter in mammalian CNS that regulates brain development and functions [202–204]. The glutamatergic system plays a crucial role in cell survival, proliferation and migration of gliomas [204]. Gln metabolizes to α-ketoglutarate and provides energy through substrate level phosphorylation in the tricarboxylic acid (TCA) cycle, thereby acting as metabolic fuel [205]. In GBM, most of the acetyl-CoA for fatty acid or lipid synthesis comes both from glycolysis and glutaminolysis [206]. A recent study has

demonstrated that GBM cells convert 90% of glucose and 60% of Gln into Lac or Ala [206]. The rapid conversion of Gln into Lac (glutaminolysis) produces sufficient NADPH to support fatty acid synthesis. Moreover, metabolomic investigations have shown that Glu release, via the cystine/glutamate antiporter system x_c^-, promotes growth of malignant gliomas. Therefore, targeting of glutamate receptors and transporters represent a novel therapeutic approach for brain tumours [202, 204].

Palanichamy and colleagues [207] have recently analysed primary and established GBM cells, GBM tissues and normal human astrocytes and identified four key metabolites (tryptophan, methionine, kynurenine, and 5-methylthioadenosine) associated with immune evasion, activation of oncogenic kinases, proliferation, survival, and development of treatment resistance in GBM cells. They propose a potential role of tryptophan-kynurenine metabolic pathway products as unique target for GBM treatment [207]. This serves as another example where amino acid depletion therapy offers exciting and promising areas of research for brain cancer.

Recently, Ha and colleagues [208] have investigated lipidomic signatures in ectopic and orthotopic human GBM xenograft models and identified glycosphingolipids, glycerophoshpoethanolamines, triacylglycerols, and glycerophosphoserines as four main classes of lipids with greatest fold effect in ectopic versus orthotopic tumour models. This study showed significant decreased of these lipid classes in brain and flank GBM tumours compared to the control brain tissue [208]. Previous lipidomic investigations have also been observed lower abundance of total lipids in malignant gliomas or grade IV astrocytoma than the low-grade astrocytomas [209, 210]. These studies suggest that in addition to glucose from anaerobic glycolysis, GBM depends on fatty acids as a fuel source [208].

MRS studies have also been conducted to distinguish between two distinct glioblastoma phenotypes named highly infiltrative non-angiogenic phenotype that shows numerous stem cell markers (low-generation, LG, tumour) and less invasive highly angiogenic phenotype (high-generation, HG, tumour) [211]. Findings of this study indicated higher concentrations of Tau, Cho and Lac in LG tumour and increased concentrations of Cho and MI with decreased concentrations of Glu and NAA in HG tumour [211]. The MRS analysis of temozolomide (TMZ) treated glioblastoma, which involve three tissue types (normal brain parenchyma, tumours treated with three TMZ cycles and untreated control tumours) was carried out to study metabolic alterations induced by treatment [212]. This study indicated lower saturated fatty acid mobile lipids (MLs) and MI/Gly ratio, and higher total ML, ML polyunsaturated fatty acid signals in treated tumour compared to untreated, and higher MLs, Cho/Cr ratio, Lac and low NAA signals in untreated tumour compared to normal brain parenchyma [212]. These metabolomics studies have proven their pivotal role in predicting glioma malignancy and prognosis.

4 Metabolomic studies in paediatric brain tumours

Most metabolomics studies in paediatric brain tumours have been conducted *in vivo* and *ex vivo*, using NMR spectroscopy. These studies have demonstrated that cell metabolites such as Cho-containing compounds, NAA, tCr, Lac, lipids, Gln/Glu and Gly are good prognostic

markers, and changes in their levels are the most sensitive parameters for differentiating paediatric brain tumours from normal tissue and monitoring disease progression [213–221].

The *ex vivo* HR-MAS NMR study conducted in 40 tissue samples from children with glial and PNET showed significantly higher Cr and Gln and lower Tau, phosphoethanolamine, PCs and Cho in glial tumour compared with PNET [215]. Another HR-MAS analysis carried out in 20 intact tissue samples from paediatric brain tumours demonstrated metabolic differences between paediatric brain tumour types, including ependymomas, medulloblastomas and pilocytic astrocytomas [218]. Results of this study showed that medulloblastoma was characterized by high levels of Cho, GPC, PC, Tau, a slightly increased level of MI, low level of fatty acids, and decreased level of NAA and Cr [218]. Pilocytic astrocytomas showed higher concentrations of fatty acids, amino acids (Ile, Leu and Val), NAA, GABA, and Glu, and lower levels of Cr, MI and Tau [218]. Paediatric ependymoma was mainly characterized by intense signals of MI and slightly lower levels of GABA, PC, and GPC compared to medulloblastoma [218].

An *in vivo* MRS study was conducted to predict treatment response of brain tumour (neuroepithelial) in children. Findings of this study demonstrated that patients who responded to chemotherapy or radiation showed higher tCr and lower Cho, Lac and lipid levels than the patients who did not respond to treatment or were not treated [219]. Also, NAA levels were low regardless of tumour state or treatment response [219]. This study suggested that tCr is the independent predictor of active tumour growth. In a similar context, MRSI analysis performed on children with neuroglial and recurrent primary brain tumours identified the change in Cho/NAA ratio as the most important prognostic indicator of tumour progression in children [220–222]. Patients with progressive paediatric brain tumours showed significantly higher percent change in Cho/NAA compared to patients with clinically stable paediatric brain tumours [220].

Moreover, an MRSI study identified elevated levels of Cho, lipids and Lac in high-grade paediatric brain tumours and proposed the metabolic signature as a predictor of clinical grades [223]. Proton-decoupled ^{31}P and ^{1}H MRSI study carried out in eight paediatric patients with untreated brain tumours and in six controls indicated significantly higher ratios of phosphoethanolamine to glycerophosphoethanolamine (PE/GPE) and PC/GPC in PNET compared with controls and other tumours [54]. The quantitative ^{1}H MRSI study indicated significantly elevated tCho concentration and reduced Cr concentration in tumours [54]. Another quantitative MRS study of untreated paediatric PNET showed elevated concentration of Tau in PNET and suggested it as the most significant metabolite for differentiation of PNET from other tumours [224].

5. Conclusion

Metabolomics studies of brain tumours highlighted in this review demonstrated that sensitive and robust metabolic profiling approaches offers great potential for a better understanding of “druggable” molecular targets/pathways and for development of novel therapeutic approaches in brain tumours. Most of the metabolomics investigations of brain tumours are based on NMR spectroscopy, especially reflecting the potential non-invasive *in*

vivo studies using MRSI. However, the low sensitivity and poor dynamic range of NMR entail that the use of highly sensitive and high throughput LC-MS techniques might be the best approach for the analysis of metabolites present at lower concentration. The high resolution and sensitive LC-MS together with NMR methods can be applied to explore signature metabolites of brain tumours in blood plasma and brain biopsies. Furthermore, study of some critical metabolic pathways such as cysteine metabolism, including GSH synthesis, and lipid metabolism can also be carried out in brain tumours, which may be helpful for discrimination of gliomas grades and the development of new strategies for clinical intervention.

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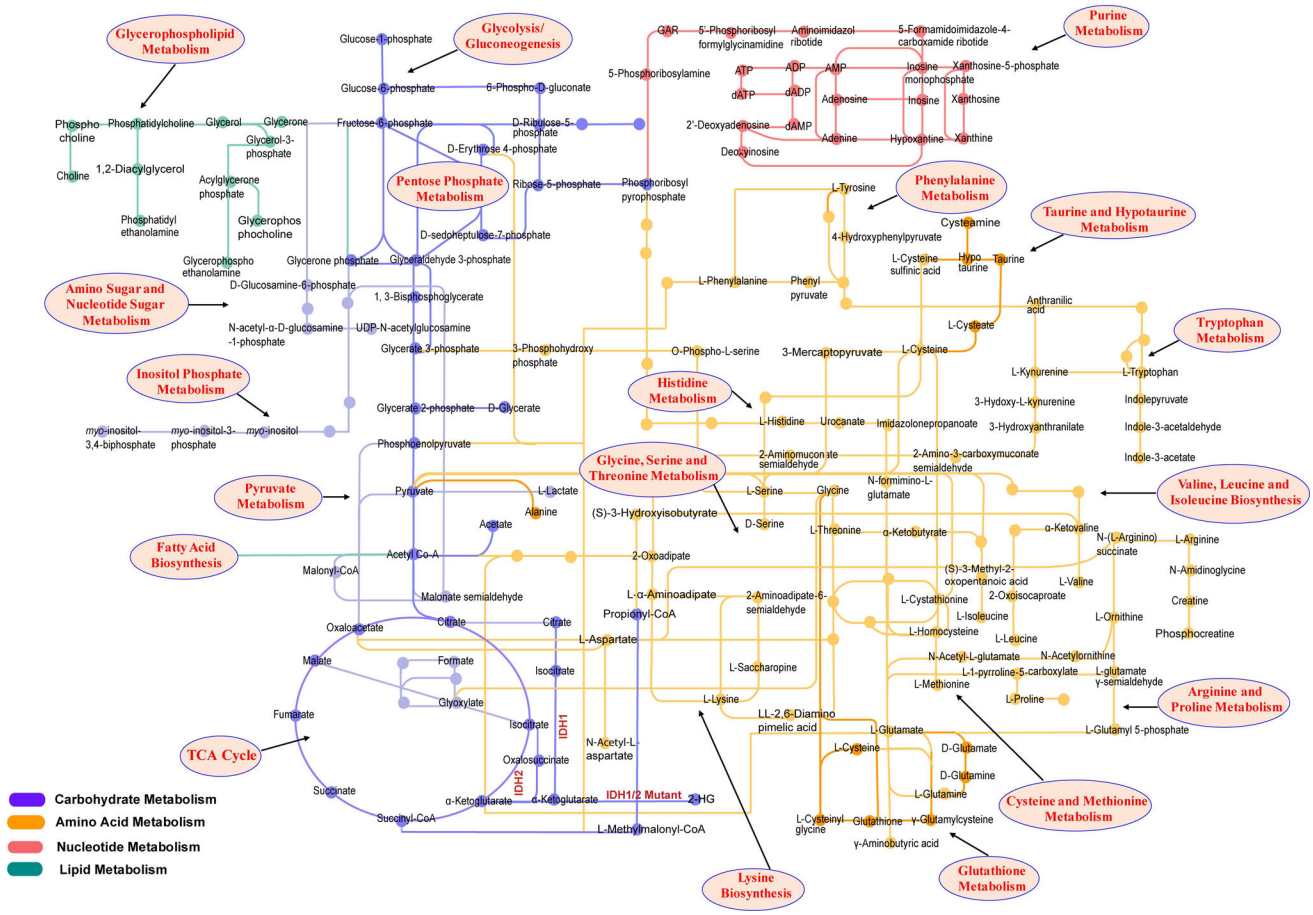

Figure 1. Major metabolomic pathways involved in brain tumour metabolism

The highlighted boxes represent the potential metabolic pathways involved in brain tumour: glycolytic intermediates, glutamine, lipids and TCA cycle metabolites significantly alter in malignant brain tumours. The accumulation of oncometabolite 2-HG resulted by IDH1/2 mutant also contributes to malignancy.

ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; dADP, deoxyadenosine diphosphate, dAMP, deoxyadenosine monophosphate; dATP, deoxyadenosine triphosphate; CoA, coenzyme A; GAR; glycinamide ribonucleotide; IDH, isocitrate dehydrogenase; UDP, uridine diphosphate

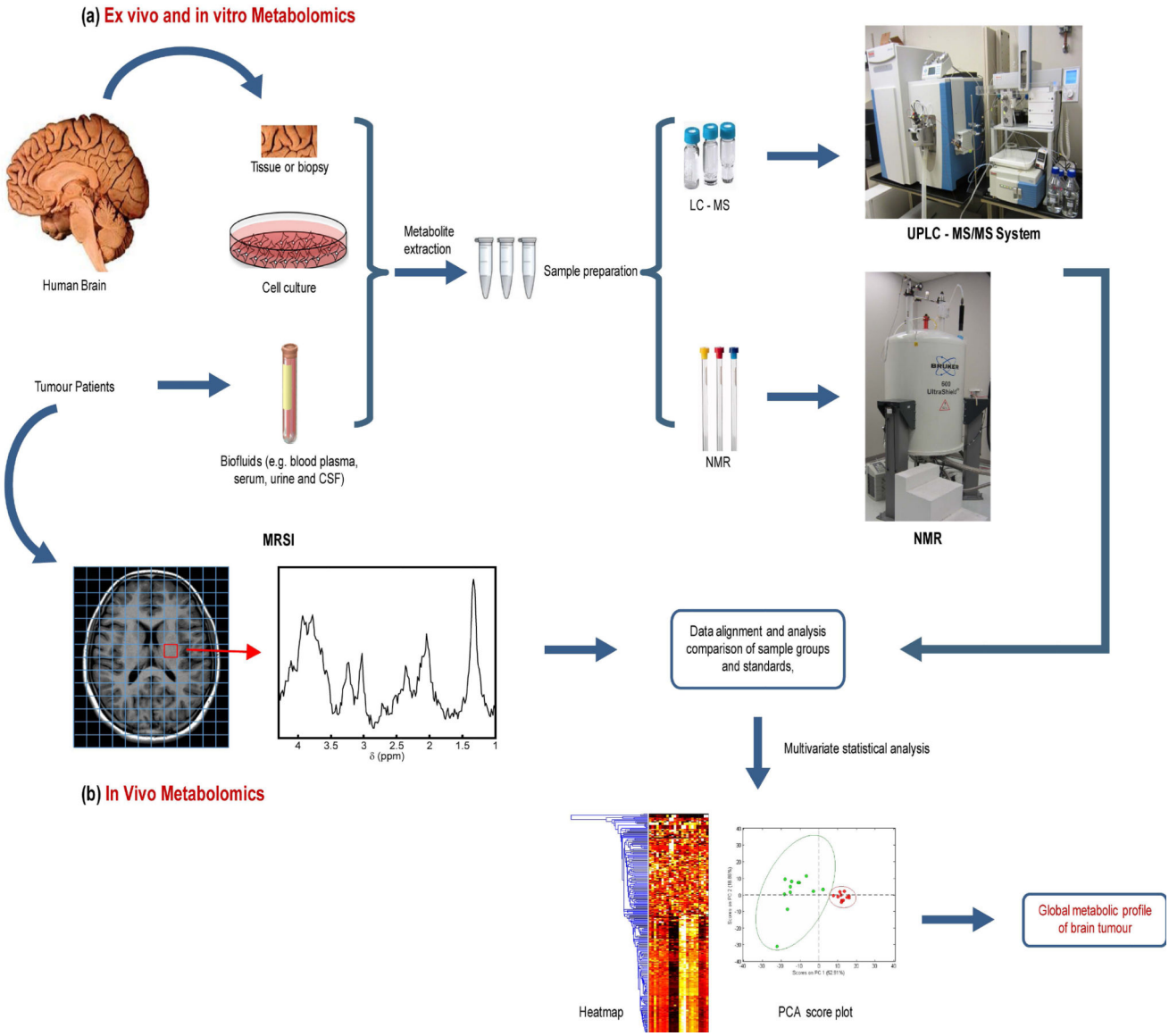

Figure 2. Workflow for brain tumour metabolomics

Metabolites are extracted from biological samples (cell, tissue, and biofluids) with the addition of internal standards using liquid-liquid extraction; polar and non-polar aliquots are dried and subsequently analysed by liquid chromatography-mass spectrometry (LC-MS) and nuclear magnetic resonance spectroscopy (NMR). The raw data is processed afterwards using metabolomics software such as XCMS and SEIVE etc. to perform peak alignment and identification. The processed data further normalized for multivariate statistical analysis. Ultimately, the workflow provides global metabolite profile and signature metabolites of different samples groups (diseased vs. healthy and treatment vs. control etc.).

Table 1

Signature metabolites detected in adult brain tumours by metabolomics.

Tumour sample source	Metabolomic study	Analytical Tool Used	Detected signature metabolites	Ref.
GBM cells vs. meningiomas and neuroblastomas	*In vitro*	^{1}HNMR & HPLC	Ala↓& Cr↓	[163]
Glioma cell lines (grade IV vs. II)	*In vitro*	^{1}HNMR	Phe↓, Cr↓, Tau↓, Lac↓, Ac↓, For↓, Val↑, Leu↑, Ile↑, Lys↑, Glu↑, Gln↑, GSH↑, GPC↑, MI↑, Thr↑& Tyr↑	[164]
GBM stem cells	*In vitro*	1D & 2D NMR	αAAD↑	[167]
Cisplatin treated GBM, medulloblastoma, ST-PNET cells	*In vitro*	^{1}HNMR & HRMAS	Uridinediphospho-N-acetylglucosamine↑& uridinediphospho-N-acetylgalactosamine↑	[165]
GBM cells	*In vitro*	GC-MS	Glucose↑, Ala↑, Asp↑& citrate↑	[166]
Plasma samples of gliomas patients vs. healthy volunteers	*Ex vivo*	^{1}HNMR & 2DNMR	Ile↓, Leu↓, Val↓, Lac↓, Ala, glycoprotein↓, Glu↓, citrate↓, Cr↓, MI↓, Cho↓, Tyr↓, Phe↓, 1-methylhistidine↓, α-glucose↓, β-glucose↓, LDL↑, unsaturated lipid↑, and pyruvate↑	[168]
Brain biopsies of HGOs vs. LGOs	*Ex vivo*	HRMAS NMR	Ala↑, Val↑, Pro↓, Gln↓, Glu↓, GABA↓, NAA↓,	[55]
Brain biopsies of GBM vs. grade II astrocytomas	*Ex vivo*	HRMAS NMR	PC↑, Gly↑, Ala↑, Tau↑, lipids↑Cr↓, MI↓, GPC↓, Cho↓& Lac↓	[169–171]
Brain biopsies of GBM vs. meningioma	*Ex vivo*	HRMAS NMR	Asp↑, Cr↑, GPC↑, His↑, MI↑, NAA↑, SI↑, Ala↓, Glu↓, GSH↓, Ile↓, Tau↓ & Val↓	[171]
Brain biopsies of GBM vs. metastasis	*Ex vivo*	HRMAS NMR	Cr↑, Gln↑, Gly↑ & H-tau↑	[171]
Brain biopsies of grade II, III astrocytomas and GBM vs. grade IV medulloblastomas	*Ex vivo*	HRMAS NMR	MI↓, Tau↓, Gly↓, PC↓, GPC↓, Asp↓, Lac↑ & Cr↑	[169]
Brain biopsies of recurrent gliomas (IV vs. II)	*Ex vivo*	HRMAS NMR	MI↓, 2-HG↑, Htau↑, Ala↑, Cho↑, lipids↑, GSH↑, PC↑ & PC/GPC↑	[172, 173]
CSF samples of malignant glioma patients (GBM vs. anaplastic astrocytomas & anaplastic oligoastrocytomas)	*Ex vivo*	LC-MS & GC-MS	Citric acid↑, isocitric acid↑, lactic acid↑, 2-aminopimelic acid↑, indole↓, indoleacrylic acid↓ & anthranilic acid↓	[174, 175]
Tissue samples from GBM vs. oligodendrogliomas	*Ex vivo*	GC-MS	Mannitol↑, Phe↑, 2-HG↓, GABA↓, Cr↓, glycerol-2-phosphate↓, glycerol-3-phosphate↓, ribitol↓ & MI↓,	[186]
Serum samples from GBM vs. oligodendrogliomas	*Ex vivo*	GC-MS	Cys↑, Lys↓ & 2-oxoisocaproic acid↓	[186]
Serum samples from GBM patients vs. healthy volunteers	*Ex vivo*	GC-MS	α-Tocopherol↑, γ-tocopherol↑, erythritol↑, MI↑, erythronic acid↑, 2-keto-L-gluconic acid↑, cystine↑ and hypoxantine↑ & xanthine↓	[215]
Urine samples from GBM patients (pre and post RT) vs. healthy volunteers	*Ex vivo*	GC-MS	N-acetylphenylalanine↑, N-acetyltryptophan, N-acetyltyrosine↑, N-acetylproline↑, TCA cycle metabolites↑, Gln↑ & N-carbamylglutamate↑	[216]
GBM cells & GBM tissues vs. normal human astrocytes	*In vitro* & *ex vivo*	LC-MS	Tryptophan↑, methionine↑, kynurenine↑ & 5-methylthioadenosine↑	[200]
HGGs vs. LGGs patients and healthy volunteers	*In vivo*	MRS	MI↑	[180]

Tumour sample source	Metabolomic study	Analytical Tool Used	Detected signature metabolites	Ref.
Brain tumour patients (gliomas) vs. healthy volunteers	*In vivo*	MRS	Cho↑, Lac↑, PC↑, Cr↓ & NAA↓,	[182–184]
GBM patients (high-generation (HG) vs. low-generation (LG) tumour)	*In vivo*	MRS	Tau↓, Cho↓& Lac↓	[201]
Primary brain tumour patients (recurrent tumour vs. postradiation change)	*In vivo*	MRS	Cho/Cr↑, Cho/NAA↑& NAA/Cr↓	[217]
TMZ treated glioblastoma patients (Treated tumour vs. untreated)	In vivo	MRS	Saturated fatty acid MLs↓, MI/Gly↓ polyunsaturated fatty acid MLs↑	[202]
(Untreated tumour vs. normal brain parenchyma)			MLs↑, Cho/Cr ↑, Lac↑ & NAA↓	

Table 2

Signature metabolites detected in paediatric brain tumours by metabolomics.

Tumour sample source	Metabolomic study	Analytical tool used	Detected signature metabolites	Ref.
Brain biopsies of glial tumour vs. PNET	*Ex vivo*	HRMAS NMR	Cr↑, Gln↑, Tau↓, PE↓, PC↓& Cho↓	[205]
Brain biopsies of pilocytic astrocytomas vs. medulloblastomas & ependymomas	*Ex vivo*	HRMAS NMR	Cr↓, MI↓, Tau↓, GPC↓, PC↓, Cho↓, NAA↑, Ile↑, Leu↑, Val↑, GABA↑, Glu↑& fatty acids↑	[208]
Neuroepithelial brain tumour patients (chemotherapy or radiation treated vs. not treated or responded)	*In vivo*	MRS	tCr↑, Cho↓, Lac↓& lipids↓	[209]
Neuroglial brain tumour patients (progressive vs. clinically stable)	*In vivo*	MRS	Cho/NAA↑	[210]
Recurrent primary brain tumour and DIPG patients (tumour vs. normal surrounding tissue)	*In vivo*	MRS	Cho:NAA↑& Cho:Cr ↑	[211, 212]
Tumour patients (high-grade vs. low-grade)	*In vivo*	MRS	Cho↑, lipids↑& Lac↑	[213]
PNET patients vs. controls and other tumors (anaplastic ependymoma, ependymoma, anaplastic astrocytoma, pilocytic astrocytoma , germinoma& fibrous histocytoma patients)	*In vivo*	^{31}P & ^{1}H MRS	PE/GPE↑, PC/GPC↑ & Tau↑	[54, 214]

↑: upregulation; ↓: downregulation; αAAD: α-aminoadipate; Ala: alanine Asp: aspartate; Cho: choline; Cr: creatine; CSF: cerebrospinal fluid; Cys: cysteine; DIPG: diffuse intrinsic pontine gliomas, GABA; γ-aminobutyrate; GBM: glioblastoma multiforme; Glu: glutamate; Gln: glutamine; GSH: glutathione; GPC: glycerophosphocholine; GPE: glycerophosphoethanolamine; Gly: glycine; HGGs: high-grade gliomas; HGOs: high-grade oligodendrogliomas; 2-HG: 2-hydroxyglutarate; His: histidine; H-tau: hypotaurine; Ile: isoleucine; Lac: lactate; Leu: leucine; LGGs: low-grade gliomas; LGOs: low-grade oligodendrogliomas; Lys: lysine; MI: *myo*-inositol; MLs: mobile lipids; NAA: N-acetyl-aspartate; PC: phosphocholine; PE: phosphoethanolamine; Phe: phenylalanine; PNET: primitive neuroectodermal tumours; Pro: proline; SI: *scyllo*-inositol; Tau: taurine; tCr: total creatine; Thr: threonine; TMZ: temozolomide; Tyr: tyrosine; Val: valine