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In vivo Deuterium Magnetic Resonance Spectroscopy reveals Glucose fluxes through glycolysis and mitochondrial oxidation pathways in mouse Glioblastoma

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Glucose fluxes in glycolytic and oxidative pathways detected in vivo by Deuterium Magnetic Resonance Spectroscopy reflect Proliferation in mouse Glioblastoma
Abstract

Objectives: Glioblastoma multiforme (GBM), the most aggressive glial brain tumors, can metabolize glucose through glycolysis and mitochondrial oxidation pathways. While specific dependencies on those pathways are increasingly associated with treatment response, detecting such GBM subtypes in vivo remains elusive. Here, we develop a dynamic glucose-enhanced deuterium spectroscopy (DGE ²H-MRS) approach for differentially assessing glucose turnover rates through glycolysis and mitochondrial oxidation in mouse GBM and explore their association with histologic features of the tumor and its microenvironment.

Materials and Methods: GL261 and CT2A glioma allografts were induced in immunocompetent mice and scanned in vivo at 9.4 Tesla, harnessing DGE ²H-MRS with volume selection and Marchenko-Pastur PCA (MP-PCA) denoising to achieve high temporal resolution. Each tumor was also classified by histopathologic analysis and assessed for cell proliferation (Ki67 immunostaining), while the respective cell lines underwent in situ extracellular flux analysis to assess mitochondrial function.

Results: MP-PCA denoising of in vivo DGE ²H-MRS data significantly improved the time-course detection (2-fold increased Signal-to-Noise Ratio) and fitting precision (-19±1% Cramér-Rao Lower Bounds) of ²H-labelled glucose, and glucose-derived glutamate-glutamine (Glx) and lactate pools in GL261 and CT2A orthotopic tumors. Kinetic modeling further indicated inter-tumor heterogeneity of glucose consumption rate for glycolysis and oxidation during a defined epoch of active proliferation in both cohorts (19±1 days post-induction), with consistent volumes (38.3±3.4 mm³) and perfusion properties prior to marked necrosis. Histopathologic analysis of these tumors revealed clear differences in tumor heterogeneity between the two GBM models, aligned with metabolic differences of the respective cell lines monitored in situ. Importantly, glucose oxidation
(i.e. Glx synthesis and elimination rates: 0.40±0.08 and 0.12±0.03 mM.min\(^{-1}\), respectively) strongly correlated with cell proliferation across the pooled cohorts (R=0.82, p=0.001; and R=0.80, p=0.002, respectively), regardless of tumor morphologic features or \textit{in situ} metabolic characteristics of each GBM model.

\textbf{Conclusions:} Our fast DGE \(^2\text{H}\)-MRS enables the quantification of glucose consumption rates through glycolysis and mitochondrial oxidation in mouse GBM, which is relevant for assessing their modulation \textit{in vivo} according to tumor microenvironment features such as cell proliferation. This novel application augurs well for non-invasive metabolic characterization of glioma or other cancers with mitochondrial oxidation dependencies.

\textbf{Keywords}

deuterium magnetic resonance spectroscopy; glioblastoma; glycolysis; mitochondrial oxidation; magnetic resonance imaging.
1. Introduction

Cancer is a highly heterogeneous disease, exhibiting multiple phenotypes that impose major challenges for clinical diagnosis and treatment, including assessment of treatment efficacy. Tumor heterogeneity can be characterized by many different aspects, such as cell proliferation, invasion, and hypoxia. However, recent observations suggest that a crucial feature of tumor heterogeneity involves the underlying metabolism (Cantor and Sabatini, 2012). While aerobic glycolysis is a well-established hallmark of cancer metabolism (Warburg effect (Warburg, 1956)), oxidation of glucose through the mitochondrial tricarboxylic acid cycle (TCA) pathway is becoming increasingly associated with microenvironment adaptation and tumor progression (Faubert et al., 2020). Such metabolic heterogeneity is observed in glioblastoma multiforme (GBM), the most aggressive form of glial brain tumors (grade IV) (Wen and Kesari, 2008). Specifically, the tumor’s proclivity to metabolize glucose through glycolysis and mitochondrial oxidation (Maher et al., 2012) is now being associated with pathway-specific dependencies for different GBM subtypes and their respective vulnerabilities to targeted treatments. Namely, a so-called mitochondrial GBM subtype, with cellular bioenergetics relying exclusively on oxidative phosphorylation (OXPHOS), has demonstrated the highest sensitivity to OXPHOS inhibition and most favorable clinical outcome (Garofano et al., 2021). Moreover, different metabolic “rewiring” of glycolysis and oxidative metabolic pathways were recently reported in subpopulations of human GBM cells (U87MG) as reflected by their temozolomide resistance, the current gold-standard for adjuvant chemotherapy in GBM (Immanuel et al., 2021). Accordingly, targeting mitochondrial metabolism for cancer therapy (Weinberg and Chandel, 2015) is currently being pursued with new treatment modalities in GBM (Molina et al., 2018; Shi et al., 2019).
Measuring metabolic fluxes in glycolysis and mitochondrial oxidation pathways simultaneously could thus compete metabolic phenotyping and early assessment of treatment efficacy. However, such characterization remains elusive due to a paucity of (invasive and non-invasive) methods with sufficient sensitivity and specificity towards properties associated with such metabolic fluxes. For example, non-invasive molecular methods such as $^{18}$FDG-PET can only detect tumor glucose uptake, but not its metabolic turnover. *In vitro* analysis of biopsy samples collected after *in vivo* administration of glucose tracers (e.g., $^{13}$C-labelled) represents an invasive approach for detecting glycolytic and oxidative metabolism of glucose but not their respective *in vivo* fluxes, in clinical and preclinical GBM tumors (Maher et al., 2012; Marin-Valencia et al., 2012). $^1$H magnetic resonance spectroscopy ($^1$H-MRS) provides noninvasive information for tumor metabolic profiling, making it possible to distinguish relevant GBM subtypes such as IHD-mut (Choi et al., 2012). However, it is unlikely that it could be used directly to assess metabolic turnover rates due to the crowded spectral areas. $^{13}$C magnetic resonance spectroscopy ($^{13}$C-MRS) can overcome this limitation to some extent by detecting the non-toxic carbon isotope in labeled glucose. Indeed, $^{13}$C-MRS with infused $^{13}$C-glucose tracers has been proposed for measuring glucose fluxes through glycolysis and mitochondrial oxidation *in vivo* in gliomas; however, these advanced methods have mostly been limited to very large voxel sizes, with significant non-tumor tissue contributions, as shown in human GBM (Wijnen et al., 2010) and patient-derived GBM xenografts (Lai et al., 2018). Furthermore, the temporal dynamic range of $^{13}$C-MRS is inherently very low due to the long carbon longitudinal relaxation constants (Lai et al., 2018).

Deuterium magnetic resonance spectroscopy ($^2$H-MRS) is a highly promising recently developed MRS modality based on intra-venous injection of non-toxic $^2$H-labelled substrates, including $^2$H-glucose. Compared to its $^1$H-MRS counterpart, $^2$H-MRS benefits from: (i) no detectable metabolic background signals, facilitating specificity and sensitivity (the natural
abundance of deuterium is very low, and typically only trace amounts are observed from the ~110M proton signal in pure water); (ii) short metabolite longitudinal relaxation times, rapid sampling of the signals; and (iii) an internal reference for quantification that does not require pre-saturation – naturally abundant semi-heavy water, DHO (Lu et al., 2017). Recent studies have demonstrated the potential of $^2$H-MRS coupled with $^2$H-glucose injection for tumor metabolic imaging, proving a non-invasive quantitative assessment of the Warburg effect in clinical and preclinical GBM (De Feyter et al., 2018) and early therapeutic response monitoring in mouse subcutaneous tumors (Kreis et al., 2020), also reported with $^2$H-fumarate injection (Hesse et al., 2021). Importantly, Dynamic Glucose-Enhanced (DGE) $^2$H-MRS has been used to measure glucose consumption rates in the normal rat brain, linked to mitochondrial oxidation (Lu et al., 2017), and glycolysis rates in a mouse lymphoma model (Kreis et al., 2020). Therefore, we hypothesized the suitability of DGE $^2$H-MRS for detecting both pathway fluxes simultaneously in GBM tumors.

Here, we selected two well-established syngeneic models of GBM – GL261 and CT2A – recapitulating histologic, genetic, and immunogenic features of the disease (Martinez-Murillo and Martinez, 2007; Oh et al., 2014; Seligman and Shear, 1939; Seyfried et al., 1992; Zagzag et al., 2000), and harnessed a novel application of DGE $^2$H-MRS with volume selection and spectral denoising based on Marchenko-Pastur PCA (MP-PCA) (Veraart et al., 2016) to: (i) demonstrate its ability to measure glucose consumption rates through glycolysis and mitochondrial oxidation in mouse GBM in vivo; and (ii) explore potential modulations of glucose metabolism according to GBM microenvironment features, such as cell proliferation (Anderson and Simon, 2020; Charles et al., 2012).
2. Materials and Methods

As summarized in Fig. 1, this study included in vivo and post-mortem assessment of mouse GBM tumors, namely the GL261 and CT2A models, which were complemented by in situ analysis of these cell lines.

Fig. 1. Experimental design. Two mouse allograft models were used to dynamically assess glucose metabolism in GBM tumors in vivo, using DGE ²H-MRS under regular anesthesia conditions: GL261 (n=7) and CT2A (n=5). GBM-bearing mice were sacrificed, and the fixed tumors further analyzed post-mortem (n=9) by histopathologic classification (H&E staining) and quantification of cell proliferation (Ki67 immunostaining). Additionally, GL261 and CT2A cancer cell metabolism were assessed in situ, during active cell proliferation in 2D culture, based on extracellular flux analysis under basal conditions followed by respiration inhibition (n=3).

2.1 Animals and cell line

All animal experiments were preapproved by institutional and national authorities and carried out according to European Directive 2010/63. A total of n=13 C57BL/6j mice were used in this study, bred at the Vivarium of the Champalimaud Foundation, and housed with ad libitum access to food and water and normal light cycles. GL261 mouse glioma cells were obtained from the Tumor Bank Repository at the National Cancer Institute (Frederick MD, USA). CT2A mouse glioma cells were kindly provided by Prof. Thomas Seyfried at Boston College (Boston MA, USA). Both cell lines were grown in RPMI-1640 culture medium supplemented with 2.0 g/l
Sodium Bicarbonate, 0.285 g/l L-glutamine, 10% Fetal Bovine Serum (Gibco) and 1% Penicillin-Streptomycin solution.

### 2.2 Mouse model of GBM

Tumors were induced in 12 mice (9 males and 3 females), as previously described (Simoes et al., 2008). Briefly, intracranial stereotactic injection of \(1 \times 10^5\) GL261 or CT2A cells was performed in the caudate nucleus; analgesia (Meloxicam 1.0 mg/Kg s.c.) was administered 30 min before the procedure. Mice were anesthetized with isoflurane (1.5-2.0% in air) and immobilized on a stereotactic holder (Kopf Instruments, Tujunga/CA, USA) where they were warmed on a heating pad at 37 ºC, monitoring the body temperature with a rectal probe (WPI ATC-2000, Hitchin, UK). The head was shaved with a small trimmer, cleaned with Betadine, and the skull exposed through an anterior-posterior incision in the midline with a scalpel. A 1 mm hole was drilled in the skull using a micro-driller, 0.1 mm posterior to the bregma and 2.32 mm lateral to the midline. The tumor cells (\(1 \times 10^5\) in 4 \(\mu\)L PBS) were inoculated 2.35 mm below the cortical surface using a 10 \(\mu\)L Hamilton syringe (Hamilton, Reno NV, USA) connected to an automatic push-pull microinjector (WPI Smartouch\textsuperscript{TM}, Sarasota FL, USA), by advancing the 26G needle 3.85 mm from the surface of the skull (~1mm skull-to-brain surface distance), pulling it back 0.5 mm, and injecting at 2 \(\mu\)L/min rate. The syringe was gently removed 2 min after the injection had finished, the skin sutured with surgical thread (5/0 braided silk, Ethicon, San Lorenzo Puerto Rico) and wiped with Betadine. The animals were kept at 25 ºC during recovery from anesthesia, and given an opioid analgesic (Buprenorphine 0.05 mg/Kg s.c.) before returning to their cage. Meloxicam analgesia was repeatedly administrated at 24- and 48-hours post-surgery.

### 2.3 in vivo imaging of GL261 tumors
2.3.1 *Longitudinal MRI*

GBM-bearing mice were imaged every 5-7 days on a 1 Tesla Icon MRI scanner (Bruker BioSpin, Ettlingen, Germany; running under *ParaVision 6.1*), to measure tumor volumes. For this, each mouse was placed in the animal holder under anesthesia (1-2 % isoflurane in 31% O₂), heated with a recirculating water blanket, and monitored for rectal temperature (36-37 ºC) and breathing (60-90 BPM). Tumor volume was measure with T2-weighted ¹H-MRI (*RARE* sequence, x8 acceleration factor, 2500 ms TR, 84 ms TE, NS 8, 1mm slice thickness, and 160 µm in-plane resolution), acquired in two orientations (coronal and axial). Each session lasted up to 30 min/animal.

2.3.2 *End-point MRI/MRS*

GBM-bearing mice were scanned 2-3 weeks post-induction on a 9.4T BioSpec MRI scanner (Bruker BioSpin, Ettlingen, Germany; running under *ParaVision 6.1*), using a customized ²H/¹H transmit-receive surface coilset (NeosBiotec, Pamplona, Spain) with a 11x15 mm inner loop (²H) and a butterfly configuration (¹H), providing quadrature B₁ field orientation to minimize coil coupling (adapted from a previous model for proton-decoupled ¹³C/³¹P spectroscopy (Adriany and Gruetter, 1997)). The coilset was initially tested *in vitro* (phantoms) to evaluate the sensitivity for dynamic and DGE ²H-MRS, and the performance of outer-volume suppression (OVS) for volume selection (*Supplementary Figs. 1 and 2*). Before each experiment, GBM-bearing mice fasted 4-6h, were weighed, and cannulated in the tail vein with a catheter connected to a home-built 3-way injection system filled with: 6,6′-²H₂-glucose (1.6M in saline); Gd-DOTA (25 mM in saline); and with heparinized saline (10 U/mL). Mice were placed on the animal holder under anesthesia (as in 2.3.1). Coilset quality factors (Q) for ¹H and ²H channels were estimated in the scanner for each sample based on the ratio of the resonance frequency (61.45 and 400.34 MHz, respectively) to its
bandwidth (full width at half-minimum of the wobbling curve during the initial tuning adjustments): 213±3 and 168±3, respectively. The mice were imaged first with T2-weighted \(^\text{1H}\)-MRI (\textit{RARE} sequence, x8 acceleration factor, 3000 ms TR, 40 ms TE; 2 averages, 1 mm slice thickness, 70 \(\mu\)m in-plane resolution) in two orientations (coronal and axial). Then, the magnetic field homogeneity was optimized over the tumor region based on the water peak with \(^\text{1H}\)-MRS (\textit{STEAM} localization: 28±2 mm\(^3\) voxel size) using localized 1\(^{\text{st}}\) and 2\(^{\text{nd}}\) order shimming with the \textit{MapShim Bruker} macro, leading to linewidths at half-maximum of 29±3 Hz (Supplementary Table 1).

\(^\text{2H}\)-MRS was performed using a pulse-acquire sequence, with 175 ms TR, 256 points, 1749 Hz, square bp RF pulse (0.128ms, 10kHz) with 55\(^{\text{o}}\) flip angle, and outer volume suppression (OVS) with 6 pulses (10 mm slabs), to excite only the tumor region according to the previously defined \textit{STEAM} voxel. After RF pulse calibration (using the natural abundance DHO peak), OVS-selective DGE \(^\text{2H}\)-MRS data were acquired for 90 min (32k repetitions), with i.v. bolus of 6,6\(^{-}\text{2H}\)-glucose (2 g/Kg, injected over 30 s; Euroisotop, St Aubin Cedex, France).

Finally, animals underwent DCE T1-weighted \(^\text{1H}\)-MRI (\textit{FLASH} sequence, 8\(^{\text{o}}\) flip-angle, 16ms TR, 4 averages, 150 repetitions, 3 slices with 1mm thickness each, 140\(\mu\)m resolution) with i.v. bolus injection of Gd-DOTA (0.1 mmol/Kg, injected over 30 s; Guerbet, Villepinte, France). After MR examination, blood glucose was measured with a glucometer (OneTouch Select Plus Flex, LifeScan, Zug, Switzerland): 8.1±1.0 mM, corresponding to euglycemia. Animals were then sacrificed under full anesthesia, their brains removed, washed in PBS, and immersed in 4\% PFA.

### 2.4. MRI/MRS processing

#### 2.4.1. \(T2\)-weighted \(^\text{1H}\)-MRI
T2-w MRI data were processed in ImageJ 1.53a (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997-2018). For each animal, the tumor region was manually delineated on each slice, and the sum of the areas multiplied by the slice thickness to estimate the volume, which was averaged across the two orientations acquired (coronal and axial). In addition, the pixel intensities from each slice were normalized to a reference region (ROI in cortex, from the contra-lateral hemisphere), and the pixel distributions for each tumor analyzed for skewness, kurtosis, and inter-quartile range (IQR).

2.4.2. DGE $^2$H-MRS

DGE $^2$H-MRS data were processed in MATLAB® R2018b (Natick, Massachusetts: The MathWorks Inc.) and jMRUI 6.0b (Stefan et al., 2009). Each dataset was averaged to 3 min temporal resolution (additional details in Supplementary Methods) and denoised with model-independent Marchenko–Pastur Principal Component Analysis (MP-PCA), taking the spectral and temporal dimensions as an M x N matrix and without any a priori constrains or assumptions, as originally reported (Veraart et al., 2016). Then, spectra were analyzed by individual peak fitting with AMARES (as before (Simoes et al., 2015)), using a basis set for DHO (4.76 ppm: short- and long-T2 fractions (De Feyter et al., 2018)) and deuterium-labelled: glucose (Glc, 3.81 ppm), glutamine-glutamate (Glx, 2.36 ppm), and lactate (Lac, 1.31 ppm); relative linewidths referenced to the estimated short-T2 fraction of DHO, according to the respective T2 relaxation times reported by de Feyter et al (De Feyter et al., 2018). The natural abundance DHO peak (DHOi) was further used to select and quantify both original and denoised spectra: SNR$_{DHOi}$ >15 and 13.88 mM reference (assuming 80 % water content in the brain and 0.03 % natural abundance of DHO), respectively. Metabolite concentrations (CRLB<50%; otherwise discarded) were then corrected for T1 and labeling-loss effects, according to the values reported by de Feyter et al (T1, ms: DHO, 320; Glc, 64; Glx, 146; Lac, 297) (De Feyter et al., 2018) and de Graaf et al (number of
magnetically equivalent deuterons: DHO, 1; Glc, 2; Glx, 1.2; Lac, 1.7) (de Graaf et al., 2021), respectively. Thus, the concentration of each metabolite \( m \) at each time point was estimated as (Eq 1):

\[
\text{Conc}_m = \frac{\text{Area}_m - \text{Area}_0}{d_m} \times \frac{C_{\text{DHO}}}{c_m} \times \frac{d_{\text{DHO}}}{\text{Area}_0} \times \text{Conc}_{\text{ref}}
\]  

\( \text{Area} \) = peak area; \( \text{Area}_0 \) = average peak area before injection; \( d \) = number of magnetically equivalent deuterons corrected for labelling-loss effects; \( C = T1 \) correction factor \((1 - \exp(-TR/T1))\); and \( \text{Conc}_{\text{ref}} \) = reference DHO concentration.

The time-course changes of \(^2\text{H}\)-labelled metabolite (Glc, Glx and Lac) concentrations were fitted using a modified version of the kinetic model reported by Kreis et al (Kreis et al., 2020), to estimate the maximum rate of Glc consumption (total, \( V_{\text{max}} \)) for Glx synthesis (mitochondrial oxidation, \( V_{\text{glx}} \)) and Lac synthesis (glycolysis, \( V_{\text{lac}} \)), and the confidence intervals for all estimated parameters:

\[
V_{\text{max}} = V_{\text{lac}} + V_{\text{glx}}
\]  

(Eq 2)

The coupled differential equations describing the concentration kinetics of each metabolite were:

\[
\frac{d[\text{Glc}]}{dt} = K_g(C_p - [\text{Glc}]) - f\left(\frac{V_{\text{max}}C_p}{f.v.k_m + C_p}\right)
\]  

(Eq 3)

\[
\frac{d[\text{Lac}]}{dt} = fV_{\text{lac}}C_p \frac{f.v.k_m + C_p}{f.v.k_m + C_p} - K_l[\text{Lac}]
\]  

(Eq 4)

\[
\frac{d[\text{Glx}]}{dt} = fV_{\text{glx}}C_p \frac{f.v.k_m + C_p}{f.v.k_m + C_p} - K_l[\text{Glx}]
\]  

(Eq 5)

where: \( k_g \), apparent rate constant of glucose transfer between blood and tumor \((\text{min}^{-1})\); \( k_{\text{glx}} \), apparent rate constant of Glx elimination \((\text{min}^{-1})\); \( k_{\text{lac}} \), apparent rate constant of lactate elimination \((\text{min}^{-1})\); \( C_p = a_1 \cdot e^{-K_p \cdot t} \), Glc concentration in plasma \((\text{mM})\); \( a_1 \), the Glc concentration after the
bolus injection (mM); $k_p$, the effective rate constant of labeled glucose transfer to tissue (min$^{-1}$); $f$, the fraction of deuterium enrichment; $v$, the extravascular-extracellular volume fraction; and $k_m$, the constant for glucose uptake. As originally reported (Kreis et al., 2020), the fraction of deuterium enrichment ($f$) and constant for glucose uptake ($k_m$) were fixed: the former, using the same estimation ($f = 0.6$, based on NMR of blood samples) since the injection protocol and dose/weight used were the same and also in mice; the latter, approximated to $k_m = 10$ mM (Marin-Hernandez et al., 2011; Williams et al., 2012). All parameters were fitted without any restrictions to their range.

2.4.3. DCE T1-weighted $^1$H-MRI

DCE T1-w MRI data were processed with DCE@urLab (Ortuno et al., 2013). ROIs were manually delineated for each slice and the time-course data was fitted with the Extended Tofts 2-compartment model (Tofts, 1997), to derive the volume transfer constant between plasma and tumor extravascular-extracellular space ($K_{\text{trans}}$), the washout rate between extravascular-extracellular space and plasma ($k_{ep}$), and the extravascular-extracellular volume fraction ($v$). The measurements were averaged across 3 slices for each tumor, covering the whole lesion, and were consistent with the literature for mouse brain tumors, e.g. $K_{\text{trans}}$ (Boult et al., 2017).

2.5. Metabolic assessment in situ

GL261 and CT2A cells were seeded overnight on Seahorse XFp miniplates (1x10$^4$ cells/well). After attachment/growth for 18 h, the RPMI$_{\text{compl}}$ medium in each well was changed to unbuffered medium (103575-100, Agilent, Santa Clara CA, USA) with the same glutamine and glucose concentration as in RPMI$_{\text{compl}}$ and incubated for 1 h in a CO$_2$-free atmosphere. The cells were then studied in situ using a Seahorse XF HS Mini Analyzer (Agilent, Santa Clara CA, USA) using the
**MitoStress Test Kit.** The MitoStress Test Kit measures the extracellular acidification rate (ECAR) and the oxygen consumption rate (OCR) in each well while specific inhibitors of the respiratory chain are sequentially injected. The data were processed with Seahorse Analytics (Agilent, Santa Clara CA, USA) and normalized to the cell number for each well. The latter were determined at the end of the experiment from the cell lysates (RIPA buffer, prepared in-house) using the bicinchoninic acid protein assay (PierceTM BCA Protein kit, ThermoFisher Scientific, Rockford IL, USA) and a microplate reader (BioTek ELMX800, Cole-Parmer, Winooski VT, USA), and assuming a total protein content 2500 µg/1 x10^7 cells, as before (Simoes et al., 2015).

### 2.6. Histopathology and Immunohistochemistry

Whole brain was fixed in 4% PFA, embedded in paraffin and sectioned at 30 different levels in its horizontal plane, spanning the whole tumor area. Sections were stained with H&E (Sigma-Aldrich, St. Louis MO, USA) and analyzed by a pathologist blinded to experimental groups. Tumor proliferation index was assessed in sections immunostained for Ki67 (mouse anti-ki67, BD, San Jose CA, USA; blocking reagent, M.O.M ImmPRESS kit, Vector Laboratories, Burlingame CA, USA; liquid DAB+, Dako North America Inc, Carpinteria CA, USA): 6-9 sections were quantified for each sample, representative of the entire tumor volume. The latter were digitized (Nanozoomer, Hamamatsu, Japan) and analyzed with QuPath 0.2.3 (https://qupath.readthedocs.io/en/latest/), blindly from DGE ²H-MRS results. Thus, the tumor regions on each slide were manually defined with ROIs, followed by semi-automated counting of Ki67⁺/⁻ cells to determine the total cell density and the labeling index (% Ki67⁺ cells). Finally, the total cell number for each tumor was estimated based on the total tumor volume (T2-w MRI data),
the average cell count per surface area (histologic counting) and assuming a cell radius of 10 µm (as reported in mouse GL261 tumors (Roberts et al., 2020)).

2.7. Statistical analyses

Data were analyzed using the two-tailed Student’s *t*-test. Differences at the 95% confidence level (p=0.05) were considered statistically significant. Correlation analyses were carried out with the Pearson R coefficient, unless indicated otherwise.
3. Results

3.1. MRI assessment of GBM allograft tumors

Orthotopic GL261 and CT2A tumors were studied in vivo 19±1 days post-injection, during a well-described epoch of active cell proliferation before marked necrosis (Cha et al., 2003; Martinez-Murillo and Martinez, 2007; Zagzag et al., 2000). Volumetric (T2-w) and DCE T1-w $^1$H-MRI of the entire cohort showed consistent tumor sizes (38.3±3.4 mm$^3$) and perfusion properties, respectively (Supplementary Table 1 and Fig. 3), in agreement with previous studies (Cha et al., 2003). Additional analysis revealed similar heterogeneity based on T2-w MRI contrast in both GBM models (skewness, kurtosis and inter-quartile range assessment of pixel distributions) but significantly higher magnetic field homogeneity achieved in CT2A tumors (-34% linewidth of $^1$H-MRS-detectable tumor water, p=0.011; leading to higher sensitivity for $^2$H-MRS detection, +23% signal-to-noise ratio of the original data, p=0.019 – Supplementary Table 1), which suggested more heterogeneous microenvironments in GL261 tumors.

3.2. Quantification of glycolytic and oxidative consumption of glucose in GBM tumors in vivo

We then performed localized DGE $^2$H-MRS (Fig. 2-A) to monitor the deuterium-labelled glucose (Glc) dynamics; namely, conversion to its downstream products lactate (Lac) and glutamate-glutamine pool (Glx) (Fig. 2-B). The location of the voxel (shown in Fig. 2-A for representative GL261 and CT2A glioma-bearing mice) was chosen such that it encompassed as much tumor volume as possible while avoiding peritumoral regions. The quality of the original spectra (Fig. 2-C upper panel) reveals good spectral resolution and the ability to distinguish between the different $^2$H-labelled metabolite pools. The signal-to-noise ratio for the natural abundance DHO peak (SNRi) in the basal original spectra was 20.0±0.9. To improve the detection
limits and kinetic profiling, we harnessed a MP-PCA denoising strategy which clearly improved the spectral quality (Fig. 2-C, lower panel). Specifically, MP-PCA indeed improved the SNRi to 43.9±3.7, demonstrating a 2-fold gain (p<0.001 in each tumor cohort) (Fig. 2-D); and an average 19±1% improvement in spectral fitting precision (Fig. 2-E) was noted. Specifically, MP-PCA denoising consistently improved the time-course detection of Glx concentration changes without altering the kinetics (Fig. 2-F).

Fig. 2. DGE $^3$H-MRS of mouse GL261 and CT2A tumors in vivo. A T2-w MRI of GL261 (G2) and CT2A (C3) tumors, displaying the DGE $^3$H-MRS volume of interest (yellow dashed line) and OVS regions (purple). B Metabolic model: Glc extravasation, cell uptake and maximum consumption rate ($V_{\text{max}}$) through glycolysis (Lac synthesis, $V_{\text{lac}}$) and mitochondrial oxidation via the TCA cycle (Glx synthesis, $V_{\text{glx}}$). C Stacked DGE $^3$H-MRS data before and after denoising (original and MP-PCA, respectively), with peak assignments. D MP-PCA denoising: left-side top, eigenvalue spectrum from PCA decomposition (blue) and fit of MP distribution (orange); right-side top, Gaussian distribution of denoising residuals, verified by the linearity of their logarithm; bottom, signal-to-noise ratio of the basal DHO peak (SNR$_{\text{DHOi}}$) before and after denoising. E Spectral fitting: top, time-domain fitting of MP-PCA spectra for absolute quantification (top - components; bottom - black, original; red, estimate; magenta, residuals), and kinetic
fitting for Glc (red), Glx (green) and Lac (blue); bottom, Cramer-Rao lower bounds of the spectral fits in the original data and after MP-PCA denoising (Glc, first 10 spectra after bolus injection; Glx and Lac, last 10 spectra). F Time-course profiles of metabolic concentrations in the original data and after MP-PCA denoising, which improved Glx detection. DHO, semi-deuterated water; Glc, 6,6′-2H₂-glucose; Glx, 4,4′-2H-glutamate-glutamine; Lac, 3,3′-2H-lactate; TCA, tricarboxylic acid. Plots: mean±SE, n=7 GL261 and n=5 CT2A. * p<0.05, ** p<0.01, *** p<0.001.

Adaptation of a previous kinetic model (Kreis et al., 2020) enabled the estimation of maximum glucose consumption rates ($V_{\text{max}}$) in GL261 tumors in vivo, for synthesis of Lac – glycolysis ($V_{\text{lac}}$) – and Glx – mitochondrial oxidation ($V_{\text{glx}}$) (Fig. 3-A). This was initially tested in parallel with two variants of the model, including the extracellular volume fraction ($v$) either as a variable ($v_{\text{estimated}}$) or fixed according to its estimation from the respective DCE T1-w MRI experiment ($v_{\text{fixed}}$), using the Extended Tofts Model (Supplementary Figure 3). While most samples (e.g. G4) consistently rendered comparable $V_{\text{glx}}$ and $V_{\text{lac}}$ estimates taking $v$ as either a model-free or model-fixed parameter, other samples (e.g. G6) did not (Fig. 3-B). Specifically, $v_{\text{estimated}}$ and $v_{\text{fixed}}$ models mostly converged to very close estimates of $V_{\text{lac}}$ and $V_{\text{glx}}$ (respectively fold-changes: 1.2±0.1 (p=0.140) and 1.1±0.1 (p=0.151), for GL261 (G1, G2, G3, G4, G5 and G7); and 1.2±0.1 (p=0.373) and 0.9±0.0 (p=0.374), for CT2A (C1,C2,C3,C4)). However, this was not verified in samples G6 and C5: while the $v_{\text{fixed}}$ model also converged to $V_{\text{lac}}$ and $V_{\text{glx}}$ estimates within the expected physiologic ranges, the $v_{\text{estimated}}$ model drifted to very large $V_{\text{lac}}$ and $v$ estimates without biological meaning, as demonstrated for the G6 sample (Fig. 3-B). Therefore, the $v_{\text{fixed}}$ approach enabled the “stabilization” of the model for all the samples in each cohort. The precision of $V_{\text{glx}}$ and $V_{\text{lac}}$ estimations with the $v_{\text{fixed}}$ model was also demonstrated (Fig. 3-C).
Fig. 3. Kinetic model for DGE $^3$H-MRS. A Modification of the model proposed by Kreis et al. (Kreis et al., 2020) for measuring the maximum rate of glucose consumption ($V_{\text{max}}$) for synthesis of lactate ($V_{\text{lac}}$) and glutamate-glutamine ($V_{\text{glx}}$): $V_{\text{max}} = V_{\text{glx}} + V_{\text{lac}}$. B Performance of $V_{\text{glx}}$ and $V_{\text{lac}}$ estimates (mM.min$^{-1}$) when $v$ (0-1) is taken as a model-free parameter of the kinetic model (model I) vs. when parameter $v$ is fixed to the value obtained from DCE T1-w data (model II), demonstrated in two tumors: top, G4; bottom, G6. Different $V_{\text{glx}}$ and $V_{\text{lac}}$ estimates were obtained by running the fitting procedures with different initial guess estimates, which were uniformly sampled between maximum/minimum values of confidence interval range across all the samples. While for some samples model I and II estimates showed comparable results (G4: 0.301 and 0.252 for $V_{\text{glx}}$, and 0.766 vs 0.702 for $V_{\text{lac}}$, respectively), in other samples meaningful estimates were only obtained by fixing $v$ to the value obtained from DCE T1-w data (G6: 0-600 and 0.117 for $V_{\text{glx}}$, and 0-1200 and 0.437 for $V_{\text{lac}}$, respectively). C Precision of the model demonstrated by iteratively (x1000) adding random noise (1%) to the parameter estimations obtained with $v$ fixed (model II) and repeating the estimations with the same model at each step. While noisy estimates from different iterations render normal distributions, their values are around the initial estimates and consistently suggesting higher $V_{\text{glx}}$ and $V_{\text{lac}}$ for G4 compared to G6 (0.252 vs. 0.177, and 0.702 vs. 0.437, respectively).

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$V_{\text{glx}}$, maximum rate of Glc consumption for Glx synthesis (mM.min$^{-1}$); $V_{\text{lac}}$, maximum rate of Glc consumption for Lac synthesis (mM.min$^{-1}$); $V_{\text{max}}$, maximum rate of total Glc consumption (mM.min$^{-1}$).
3.3. Heterogeneity of glucose metabolism in GBM tumors \textit{in vivo}

Harnessing the kinetic model with extracellular volume fixed to that measured from DCE, the estimations of $V_{\text{lac}}$ ($0.54\pm0.07$ mM.min$^{-1}$) and $V_{\text{glx}}$ ($0.40\pm0.08$ mM.min$^{-1}$) in GBM allograft tumors could be robustly extracted (Figs. 3, 4). Marked inter-tumor metabolic heterogeneity in glucose consumption rate through glycolysis and mitochondrial oxidation, respectively, was clearly demonstrated (Fig. 4). Thus, while some tumors appeared to rely mostly on glycolytic turnover of glucose (e.g. G3, $V_{\text{lac}}=0.46\pm0.1$ and $V_{\text{glx}}=0.02\pm0.01$ mM.min$^{-1}$), other tumors ranged up to similar glucose consumption rates through each pathway flux (e.g. C4, $V_{\text{lac}}=0.44\pm0.5$ and $V_{\text{glx}}=0.52\pm0.05$ mM.min$^{-1}$; ).

![Fig. 4. Glucose consumption rates in mouse GBM tumors. Estimated maximum rates of glucose consumption in GL261 (A) and CT2A (B) tumors, for de novo synthesis of (estimate±SE mM.min$^{-1}$): Glx, 0.40±0.08 ($V_{\text{glx}}$, green); Lac, 0.54±0.07 ($V_{\text{lac}}$, blue); and total, 0.94±0.13 ($V_{\text{max}}$, red).](image)

3.4. Functional assessment of mitochondrial respiration and glycolysis in glioma cell lines \textit{in situ}

To investigate whether our \textit{in vivo} findings were related to basal metabolic properties of each tumor model, additional \textit{in situ} analysis were performed for functional metabolic assessment of
the respective cell lines. Specifically, we investigated glycolysis and mitochondrial oxidation metabolism at the cellular level during active proliferation in 2D cell culture (Fig. 5-A). The results clearly indicated simultaneous mitochondrial OXPHOS (Fig. 5-B) and glycolytic metabolism (Fig. 5-C) under regular growth conditions (basal respiration, Fig. 5-D-E) in GL261 and CT2A cells – namely, 4.13±0.52 and 2.67±0.49 fmol O₂ min⁻¹.cell⁻¹ OCR (respectively), within the range of literature values reported with primary GBM cells and other GBM cell lines (Arthurs et al., 2020). While the results indicate oxygen consumption mostly coupled to ATP production in both cell lines (>65% of basal respiration, Fig. 5-D), GL261 and CT2A cells revealed marked differences in mitochondrial function. Thus, GL261 cells had a significant respiration buffer (maximal and spare respiration capacity ~5-fold higher than basal respiration, Fig. 5-D), not observed in CT2A cells. In addition, only GL261 cells demonstrated precise increases of glycolytic flux during stepwise inhibition of OXPHOS (Fig. 5-E – basal ECAR measurements also within the range of previous studies in primary GBM cells and other GBM cell lines (Arthurs et al., 2020)), revealing an efficient metabolic plasticity.
Fig. 5. **Functional metabolic assessment of GL261 and CT2A cells in situ.** A Extracellular flux analysis of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in glioma cells proliferating on micro-well plates during stepwise inhibition of OXPHOS: mitochondrial ATP synthase by oligomycin (dark blue), mitochondrial uncoupling by FCCP (yellow), and complex I by rotenone/antimycin-A (dark red). B-E Time-course monitoring of OCR (B) and ECAR (C) and respective parameters derived for mitochondrial OXPHOS (D) and glycolysis (E). Average cell count estimates in each well (x10⁻³ cells.µm⁻²): GL261, 3.44±1.49; CT2A, 5.50±1.38. Plots: mean±SE (n=3); * p<0.05, ** p<0.01 vs. basal condition; # p<0.001 (grey p<0.05) vs. GL261.

3.5. **Histopathology of GBM tumors**

Given that our *in situ* findings could suggest differential metabolic adaptability of GL261 and CT2A cells to microenvironmental changes during GBM proliferation, potentially consistent with the heterogeneity of glucose metabolic fluxes detected *in vivo* (**Fig. 4**), histopathological analysis was performed. Tumors were screened for cytomorphological features, cell density, presence of hemorrhage, necrosis, and for cell proliferation index, blinded to *in vivo* MRI/MRS data (**Fig. 6**). While no significant difference was seen in extent of necrosis between GL261 and CT2A tumors, only the latter displayed extensive morphologic heterogeneity (**Fig. 6-A, Supplementary Table 2**), in agreement with the differences in magnetic field homogeneity detected *in vivo* in each model (**Supplementary Table 1**). Specifically, GL261 tumors showed distinct morphological features associated with progressive compression by the expansile tumor growth, including compression of vasculature, damage to the vessels, hemorrhage and edema. Tumors were scored individually for the following stromal-vascular phenotype: I, small vessels, complete endothelial cell lining and sparse hemorrhages; II, vasodilation and marked multifocal hemorrhages; III, necrosis of the vascular wall, incomplete endothelial cell lining, vascular leakage, and edematous stroma; and IV, vascular depletion and edematous stroma (**Supplementary Fig. 4**). Further assessment of cell proliferation index based on ki67 immunostaining (**Fig. 6-B**) indicated a strong correlation of this
parameter with the stromal-vascular fraction scores in GL261 tumors (Kendall’s rank coefficient: \(\tau=0.80, p=0.017\)), which was not observed in CT2A tumors (Fig. 6-C). The latter displayed 2-fold higher cellularity than GL261 tumors (Fig. 6-C; 8.7±0.2 vs 4.0±0.2 \(\times 10^8\) cells.mL\(^{-1}\), \(p<0.0001\) – Supplementary Table 1) and a remarkable homogeneity of cell morphology, with small vessels, complete endothelial cell lining and sparse hemorrhages, consistent with a stromal-vascular phenotype score I (Supplementary Table 2).

Fig. 6. Histopathologic analyses of GBM tumors. A Representative microphotographs of H&E-stained CT2A (I, C3) and GL261 (ii, G2) brain tumors, showing well circumscribed lesions (black arrowhead) of similar size, in the same anatomical location, adjacent to and compressing the hippocampus (hpc). The tumors show distinct morphological features: CT2A are composed by dense, cohesive and homogeneous tumor cell population (i’), rich in small vessels with preserved integrity (white arrow) (ii’’); while GL261 tumors show marked heterogeneity, with poorly cohesive areas and marked intercellular edema (black arrow) (ii), that intercalate with large dilated vessels, occasionally with loss of endothelial lining (white arrowhead) and extensive hemorrhages (ii). The various phenotypes encountered in the stromal-vascular fractions of these tumors are described in detail in Supplementary Fig. 4. B Ki67 immunostaining of a brain section (G2) displaying the tumor ROI for quantification (yellow-line), with enlarged view of a sub-tumor region (white-rectangle) showing Ki67\(^+\) (brown) and Ki67\(^-\) (blue) cells, and respective counting (mean cell densities: GL261, 4.0±0.2; CT2A, 8.6±0.2 \(\times 10^3\) cells.\(\mu\)m\(^{-2}\)). C Cell detection measurements in 7.0±0.4 histologic
sections for each tumor (means±SE displayed), showing significantly higher density in CT2A tumor within the same proliferation range. Legend: black-arrow, small vessels; white-arrow, vasodilation; asterisk, multifocal hemorrhages; black-arrowhead, incomplete endothelial cell lining; cardinal, edematous stroma white-arrowhead, edematous stroma. *** p<0.0001.

3.6. Modulation of glucose metabolism according to cell proliferation in GBM tumors

Finally, we explored potential associations between glucose metabolic heterogeneity and tumor microenvironment features in GBM allografts (Fig 7-A). Thus, glucose consumption rate correlated significantly with cell proliferation index across the pooled cohorts (n=12) and regardless of the histopathologic phenotype (R=0.71, p=0.010 – Fig. 7-A). Importantly, this was associated specifically with glucose mitochondrial oxidation (i.e. Glx synthesis and elimination rates: R=0.82, p=0.001; and R=0.80, p=0.002, respectively – Fig. 7-B and 7-C) rather than glycolysis (Fig. 7-D and 7-E), which was already detectable in the more heterogeneous GL261 model (R=0.84, p=0.018; and R=0.84, p=0.019, respectively – Supplementary Table 1). Moreover, no association was observed between cell proliferation and other tumor parameters, such as volume, perfusion, heterogeneity based on T2w-MRI contrast, histopathologic phenotype, or cell density (Supplementary Table 1).
Fig. 7. Association between glucose metabolism and cell proliferation in pooled GL261 and CT2A tumor cohorts. Cell proliferation index correlated significantly with glucose consumption rate (A), which was associated with its mitochondrial oxidation, i.e. rates of Glx synthesis (B) and elimination (C), rather than glycolytic turnover, i.e. rates of Lac synthesis (D) or elimination (E). Plots: estimate±CI for glucose metabolism-derived metrics, and means±SE for cell proliferation index.
4. Discussion

The ability of DGE $^2$H-MRS to quantify glucose metabolic rates through mitochondrial oxidation (normal rat brain (Lu et al., 2017)) or glycolysis (subcutaneous mouse tumors (Kreis et al., 2020)), has been rapidly gaining interest for in vivo metabolic flux assessment. Despite the great promise for in vivo DGE $^2$H-MRS, the typically low signal-to-noise ratio in these experiments, which is mainly incurred due to deuterium’s low resonance frequency (compared with its $^1$H counterpart), imposes significant boundaries on their ability to characterize the relevant metabolic rates and/or accurately define relatively weak signals, such as Glx. Thus, simultaneous characterization of both glycolytic and oxidative pathways remains limited. This work aimed to harness the DGE $^2$H-MRS methodology with volume selection and MP-PCA spectral denoising to overcome these major limitations, and investigate whether such simultaneous measurements could address the current need to detect specific metabolic pathway-dependencies in GBM tumors (Garofano et al., 2021). Furthermore, we investigated the potential relevance of DGE $^2$H-MRS for characterizing the links between metabolic heterogeneity and histopathologic features, including cell proliferation.

To test these features, we used two robust, immunocompetent mouse models mimicking clinical GBM – GL261 and CT2A (Griguer et al., 2005; Martinez-Murillo and Martinez, 2007; Oh et al., 2014; Seligman and Shear, 1939; Seyfried et al., 1992; Zagzag et al., 2000) – which evidenced pronounced histologic and metabolic heterogeneity and thereby were suitable for testing our hypotheses. Our three-pronged strategy was designed to: (i) measure the in vivo glucose metabolic rates in orthotopic GBM tumors using DGE $^2$H-MRS and a novel unbiased denoising strategy (MP-PCA) that increases the confidence and robustness of the fits; (ii) contrast these metrics with functional metabolic assessment of the respective cell lines under controlled in situ
conditions; and (iii) assessing the tumor heterogeneity via unbiased histopathologic and cell proliferation assessment.

The *in vivo* DGE $^2$H-MRS-driven estimates of glycolytic flux in GL261 tumors ($V_{lac}$: GL261, $0.50\pm0.07$ mM.min$^{-1}$ or $1.25\pm0.16$ fmol.min$^{-1}$.cell$^{-1}$; CT2A, $0.60\pm0.13$ mM.min$^{-1}$ or $0.70\pm0.16$ fmol.min$^{-1}$.cell$^{-1}$) were within the ranges reported in the literature for other tumor models, such as: mouse lymphoma, also using *in vivo* DGE $^2$H-MRS ($0.99$ mM.min$^{-1}$ (Kreis et al., 2020)); rat breast cancer xenografts, based on biochemical assay analysis of tissue-isolated samples ($1.43$ mM.min$^{-1}$ (Kallinowski et al., 1988), assuming $1\text{mL}\sim 1.1\text{g}$); and even perfused U87 GBM cells, using *in situ* hyperpolarized $^{13}$C-MRS ($\sim 1.35$ fmol.min$^{-1}$.cell$^{-1}$ (Jeong et al., 2017)). With regards to glucose consumption rate through mitochondrial oxidation ($V_{glx}$: GL261, $0.32\pm0.10$ mM.min$^{-1}$ or $0.77\pm0.23$ fmol.min$^{-1}$.cell$^{-1}$; CT2A, $0.51\pm0.11$ mM.min$^{-1}$ or $0.60\pm0.12$ fmol.min$^{-1}$.cell$^{-1}$), our estimates were closely consistent with the *in situ* results in cell culture (up to $0.69\pm0.09$ and $0.44\pm0.08$ fmol.min$^{-1}$.cell$^{-1}$, respectively – assuming a $1\text{ Glc} : 6\text{ O}_2$ stoichiometry to fully sustain OXPHOS), thereby validating the robustness of these *in vivo* measurements towards estimating glucose metabolic fluxes. Moreover, although the kinetic model used is rather simplistic compared to more established ones for assessing cerebral metabolic rates of glucose consumption in healthy rodent brain with labelled tracers, including deuterated glucose ($0.25$ mM.min$^{-1}$ (Lu et al., 2017)), our findings are also well aligned with prior $^{13}$C-MRS-based estimates in mouse GBM xenografts ($0.33$ mM.min$^{-1}$ (Lai et al., 2018)), lending further credence to this strategy.

Assessment of mitochondrial function *in situ* further suggested the coupling between basal respiration and energy production in both glioma cell lines. While this should be taken with caution considering glutamine-driven mitochondrial substrate level phosphorylation in the glutaminolysis pathway for ATP synthesis in several cancer cells, including CT2A (Chinopoulos and Seyfried, 2018; Duraj et al., 2021), both approaches link ATP production to mitochondrial metabolism.
More importantly, there were marked metabolic differences between the two cell lines, consistent with different sensitivities to treatment previously reported (McKelvey et al., 2021), and distinct histopathologic features of the respective tumors. GL261 cells demonstrated strong respiration buffer capacity (up to 5-times the basal respiration rate) and efficient metabolic plasticity between glycolysis and mitochondrial oxidation, generating orthotopic tumors with heterogeneous stromal-vascular phenotypes (scores I-IV) closely reflecting their cell proliferation index, which in turn correlated strongly with glucose mitochondrial oxidation in vivo. Thus, despite the instability and disruption of tumor-associated vessels toward more aggressive histopathologic phenotypes, the overall good perfusion of non-necrotic GL261 tumors in vivo should support the oxygen demands for increasing mitochondrial metabolism, likely to sustain the high anabolic and energetic requirements for cell proliferation while protecting against reactive oxygen species in the hemorrhagic stroma (Weinberg et al., 2019).

Interestingly, while the glucose oxidation fraction ($V_{\text{glx}}/V_{\text{max}}$) was significantly higher in GL261 tumors with more aggressive phenotype (IV vs I-II-III, $p=0.026$) under regular anesthesia conditions (fraction of inspired oxygen ($\text{FiO}_2$), 31%; blood oxygen saturation ($\text{SpO}_2$), 98.8±0.2%), preliminary data with additional GL261 tumors indicated decreased glucose oxidation fraction under acute hypoxia (FiO$_2$, 12%; SpO$_2$, 61.9±1.6% – Supplementary Fig. 5 and Supplementary Tables 2 and 3). This was consistent with the metabolic plasticity demonstrated in situ during OXPHOS inhibition and suggests quick metabolic adaptation to lower oxygen tensions, typically present in advanced malignant tumors. Accordingly, metabolic plasticity represents a key element for cancer cell survival, adaptation, and proliferation in a rapidly shifting microenvironment (Fendt et al., 2020; Gillies et al., 2012; Lehuede et al., 2016), with a pivotal role for mitochondrial reprogramming between e.g. invasive and proliferative states (the latter supported by oxidative metabolism (Li et al., 2020)), which are present in GBM (Rajapakse et al., 2020; Xie et al., 2014).
Altogether, our observations strengthen the relevance of DGE $^2$H-MRS for in vivo detection of GBM dependencies on oxidative metabolism at any given progression stage. This could be helpful for early treatment assessment, by evaluating the response to: antiangiogenic therapies, which impact tumor perfusion (oxygenation) and therefore the ability to rely on oxidative metabolism (Batchelor et al., 2014); OXPHOS-targeted treatments (Molina et al., 2018; Shi et al., 2019); or even the efficiency of chemosensitization to those or other therapies, e.g. with dichloroacetate (Michelakis et al., 2010; Shen et al., 2015).

Compared to the GL261 model, CT2A cells demonstrated more limited metabolic flexibility in situ. Namely, CT2A cells displayed markedly reduced respiration buffer capacity and no apparent glycolytic response to acute inhibition of OXPHOS. This was consistent with a conserved, less aggressive stromal-vascular phenotype (score I) of CT2A tumors, and 2-fold higher cellular density than GL261’s. Despite such metabolic and histopathologic differences between the two allograft models, pooling them strengthen our previous finding that non-necrotic GBM tumors with consistent sizes and perfusion properties had an increasing reliance on glucose mitochondrial oxidation according to cell proliferation index. This is aligned with recent observations of increasing OXPHOS-dependance at more advanced stages of tumor progression (Faubert et al., 2020), and even polarization of tumor-associated macrophages towards an OXPHOS-dependent, pro-tumorigenic M2 phenotype (Van den Bossche et al., 2017). Thus, our results demonstrate the potential of DGE $^2$H-MRS for non-invasive detection of clinically relevant GBM phenotypes.

As in every study, we acknowledge the limitations of this work. Firstly, although the Glx peak region was assigned to the glutamate-glutamine pool, this region overlaps with other TCA-cycle intermediates/derivatives such as succinate (2.39 ppm), which has been detected e.g. in breast cancer cell lines with TCA-cycle truncations (Simoes et al., 2015). While no such truncations have
been reported in the GL261 model, and are not expected according to the \textit{in situ} Seahorse experiments performed, the CT2A model could potentially harbor them given the electron transport chain abnormalities reported (Kiebish et al., 2008). In any case, the Glx region assigned in DGE $^2$H-MRS should still reflect \textit{de novo} mitochondrial turnover of glucose. Secondly, the normal variations in tumor shape within each cohort led to some discrepancies in the DGE 2H-MRS voxel vs. total tumor volumes. Specifically, tumors G4 and C4 – the smallest in their respective cohorts – presented more regular shapes that mostly fitted the total voxel volume. While this led to $\sim$20\% larger voxel volume than total volume, the same tumor/non-tumor proportion was kept during visual adjustment of the voxel in these tumors (as in all others); otherwise, lower rates of glycolysis vs glucose oxidation would be expected in these samples due to stronger contaminations from non-tumor tissue, which was not the case (Supplementary Table 1, Vlac/Vglx: G4$>$G2$\sim$G1$>$G7$\sim$G5; C4$\sim$C1$>$C2). Moreover, the differences in voxel vs tumor volumes did not correlate significantly to glucose oxidation or glycolytic rates within each cohort or across pooled cohorts (Supplementary Table 4), further suggesting no association between those variables, we performed the study at 9.4 Tesla, which represents a higher magnetic field strength compared to standard clinical scanners (1.5 to 3.0 Tesla). However, since deuterium spectroscopy and imaging (De Feyter et al., 2018) benefit significantly from increased field strengths, their translational application to the human brain has already been demonstrated at 7.0 Tesla (de Graaf et al., 2020) and could be extended to 9.4 Tesla human scanners.
5. Conclusion

This study demonstrated (i) the potential of DGE $^2$H-MRS for \textit{in vivo} assessment of glucose consumption rates through glycolytic and oxidative pathways simultaneously in mouse GBM, and potentially other aggressive tumors with OXPHOS dependencies (Mendez-Lucas et al., 2020); and (ii) its relevance for metabolic characterization of GBM, as evidenced here by the strong association between the heterogeneity of glucose consumption rates and cell proliferation in two well-established allograft models prior to marked necrosis. The relevance of this novel application for non-invasive stratification and early assessment of treatment efficacy in GBM patients (Garofano et al., 2021) warrants its extension to additional tumor models and progression stages, and even patients. Given that clinical translation of deuterium imaging has already been demonstrated for GBM (De Feyter et al., 2018), and treatment-response monitoring with DGE deuterium spectroscopy and imaging reported in a mouse lymphoma model (Kreis et al., 2020), our findings augur well for such future applications of DGE $^2$H-MRS in research and in the clinic.
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Appendix A. Supplementary Data

SimoesRV_SupplementaryData.pdf
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FIGURES

Figure 1. Experimental design. Two mouse allograft models were used to dynamically assess glucose metabolism in GBM tumors in vivo, using DGE $^2$H-MRS under regular anesthesia conditions: GL261 (n=7) and CT2A (n=5). GBM-bearing mice were sacrificed, and the fixed tumors further analyzed post-mortem (n=9) by histopathologic classification (H&E staining) and quantification of cell proliferation (Ki67 immunostaining). Additionally, GL261 and CT2A cancer cell metabolism were assessed in situ, during active cell proliferation in 2D culture, based on extracellular flux analysis under basal conditions followed by respiration inhibition (n=3).

Figure 2. DGE $^2$H-MRS of mouse GL261 and CT2A tumors in vivo. A T2-w MRI of GL261 (G2) and CT2A (C3) tumors, displaying the DGE $^2$H-MRS volume of interest (yellow dashed line) and OVS regions (purple). B Metabolic model: Glc extravasation, cell uptake and maximum consumption rate ($V_{max}$) through glycolysis (Lac synthesis, $V_{lac}$) and mitochondrial oxidation via the TCA cycle (Glx synthesis, $V_{glx}$). C Stacked DGE $^2$H-MRS data before and after denoising (original and MP-PCA, respectively), with peak assignments. D MP-PCA denoising: left-side top, eigenvalue spectrum from PCA decomposition (blue) and fit of MP distribution (orange); right-side top, Gaussian distribution of denoising residuals, verified by the linearity of their logarithm; bottom, signal-to-noise ratio of the basal DHO peak (SNR$_{DHOi}$) before and after denoising. E Spectral fitting: top, time-domain fitting of MP-PCA spectra for absolute quantification (top - components; bottom - black, original; red, estimate; magenta, residuals), and kinetic fitting for Glc (red), Glx (green) and Lac (blue); bottom, Cramer-Rao lower bounds of the spectral fits in the original data and after MP-PCA denoising (Glc, first 10 spectra after bolus injection; Glx and Lac, last 10 spectra). F Time-course profiles of metabolic concentrations in the original data and after MP-PCA denoising, which improved Glx detection. DHO, semi-deuterated water; Glc, 6,6$^{'-}$H$_2$-glucose; Glx, 4,4$^{'-}$H-glutamate-glutamine; Lac, 3,3$^{'-}$H-lactate; TCA, tricarboxylic acid. Plots: mean±SE, n=7 GL261 and n=5 CT2A. * p<0.05, ** p<0.01, *** p<0.001.

Figure 3. Kinetic model for DGE $^2$H-MRS. A Modification of the model proposed by Kreis et al. (Kreis et al., 2020) for measuring the maximum rate of glucose consumption ($V_{max}$) for synthesis
of lactate \((V_{\text{lac}})\) and glutamate-glutamine \((V_{\text{glx}})\): \(V_{\text{max}} = V_{\text{glx}} + V_{\text{lac}}\). B Performance of \(V_{\text{glx}}\) and \(V_{\text{lac}}\) estimates (mM.min\(^{-1}\)) when \(v\) (0-1) is taken as a model-free parameter of the kinetic model (model I) vs. when parameter \(v\) is fixed to the value obtained from DCE T1-w data (model II), demonstrated in two tumors: top, G4; bottom, G6. Different \(V_{\text{glx}}\) and \(V_{\text{lac}}\) estimates were obtained by running the fitting procedures with different initial guess estimates, which were uniformly sampled between maximum/minimum values of confidence interval range across all the samples. While for some samples model I and II estimates showed comparable results (G4: 0.301 and 0.252 for \(V_{\text{glx}}\), and 0.766 vs 0.702 for \(V_{\text{lac}}\), respectively), in other samples meaningful estimates were only obtained by fixing \(v\) to the value obtained from DCE T1-w data (G6: 0-600 and 0.117 for \(V_{\text{glx}}\), and; 0-1200 and 0.437 for \(V_{\text{lac}}\), respectively). C Precision of the model demonstrated by iteratively (x1000) adding random noise (1%) to the parameter estimations obtained with \(v\) fixed (model II) and repeating the estimations with the same model at each step. While noisy estimates from different iterations render normal distributions, their values are around the initial estimates and consistently suggesting higher \(V_{\text{glx}}\) and \(V_{\text{lac}}\) for G4 compared to G6 (0.252 vs. 0.177, and 0.702 vs. 0.437, respectively). 

\(a_i\), Glc concentration after the bolus injection (mM); \(k_p\), effective rate constant of labeled glucose transfer to tissue (min\(^{-1}\)); \(k_g\), apparent rate constant of glucose transfer between blood and tumor (min\(^{-1}\)); \(V_{\text{glx}}\), maximum rate of Glc consumption for Glx synthesis (mM.min\(^{-1}\)); \(k_{\text{glx}}\), apparent rate constant of Glx elimination (min\(^{-1}\)); \(V_{\text{lac}}\), maximum rate of Glc consumption for Lac synthesis (mM.min\(^{-1}\)); \(k_{\text{lac}}\), apparent rate constant of lactate elimination (min\(^{-1}\)); \(V_{\text{max}}\), maximum rate of total Glc consumption (mM.min\(^{-1}\)).

**Figure 4. Glucose consumption rates in mouse GBM tumors.** Estimated maximum rates of glucose consumption in GL261 (A) and CT2A (B) tumors, for \textit{de novo} synthesis of (estimate±SE mM.min\(^{-1}\)): Glx, 0.40±0.08 \((V_{\text{glx}}, \text{green})\); Lac, 0.54±0.07 \((V_{\text{lac}}, \text{blue})\); and total, 0.94±0.13 \((V_{\text{max}}, \text{red})\).

**Figure 5. Functional metabolic assessment of GL261 and CT2A cells \textit{in situ}.** A Extracellular flux analysis of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in glioma cells proliferating on micro-well plates during stepwise inhibition of OXPHOS: mitochondrial ATP synthase by oligomycin (dark blue), mitochondrial uncoupling by FCCP (yellow), and complex I by rotenone/antimycin-A (dark red). B-E Time-course monitoring of OCR (B) and ECAR (C) and respective parameters derived for mitochondrial OXPHOS (D) and glycolysis (E). Average cell count estimates in each well \((x10^3 \text{cells.µm}^{-2})\): GL261, 3.44±1.49; CT2A, 5.50±1.38. Plots: mean±SE \((n=3)\); * \(p<0.05\), ** \(p<0.01\) vs. basal condition; # \(p<0.001\) (grey \(p<0.05\)) vs. GL261.

**Figure 6. Histopathologic analyses of GBM tumors.** A Representative microphotographs of H&E-stained CT2A (I, C3) and GL261 (ii, G2) brain tumors, showing well circumscribed lesions (black arrowhead) of similar size, in the same anatomical location, adjacent to and compressing
the hippocampus (hpc). The tumors show distinct morphological features: CT2A are composed by dense, cohesive and homogeneous tumor cell population (i’), rich in small vessels with preserved integrity (white arrow) (ii’’); while GL261 tumors show marked heterogeneity, with poorly cohesive areas and marked intercellular edema (black arrow) (ii), that intercalate with large dilated vessels, occasionally with loss of endothelial lining (white arrowhead) and extensive hemorrhages (ii). The various phenotypes encountered in the stromal-vascular fractions of these tumors are described in detail in Supplementary Fig. 4. C Ki67 immunostaining of a brain section (G2) displaying the tumor ROI for quantification (yellow-line), with enlarged view of a sub-tumor region (white-rectangle) showing Ki67+ (brown) and Ki67− (blue) cells, and respective counting (mean cell densities: GL261, 4.0±0.2; CT2A, 8.6±0.2 x10^{-3} cells.µm^{-2}). D Cell detection measurements in 7.0±0.4 histologic sections for each tumor (means±SE displayed), showing significantly higher density in CT2A tumor within the same proliferation range. Legend: black-arrow, small vessels; white-arrow, vasodilation; asterisk, multifocal hemorrhages; black-arrowhead, incomplete endothelial cell lining; cardinal, edematous stroma white-arrowhead, edematous stroma. *** p<0.0001.

Figure 7. Association between glucose metabolism and cell proliferation in pooled GL261 and CT2A tumor cohorts. Cell proliferation index correlated significantly with glucose consumption rate (A), which was associated with its mitochondrial oxidation, i.e. rates of Glx synthesis (B) and elimination (C), rather than glycolytic turnover, i.e. rates of Lac synthesis (D) or elimination (E). Plots: estimate±CI for glucose metabolism-derived metrics, and means±SE for cell proliferation index.
SUPPLEMENTARY TABLES

Supplementary Table 1. Mouse GBM tumor. Animal information and metrics obtained from multi-modal in vivo MRI/MRS and post-mortem histopathology and immunostaining.

$K_{\text{trans}}$, volume transfer constant between plasma and tumor extravascular-extracellular space; $k_e p$, washout rate between extravascular-extracellular space and plasma; $v$, extravascular-extracellular volume fraction; WL, water linewidth at half-maximum of the water peak; $a1$, Glc concentration after the bolus injection (mM); $k_p$, effective rate constant of labeled glucose transfer to tissue (min$^{-1}$); $k_g$, apparent rate constant of glucose transfer between blood and tumor (min$^{-1}$); $V_{\text{glx}}$, maximum rate of Glc consumption for Glx synthesis (mM. min$^{-1}$); $k_{\text{glx}}$, apparent rate constant of Glx elimination (min$^{-1}$); $V_{\text{lac}}$, maximum rate of Glc consumption for Lac synthesis (mM. min$^{-1}$); $k_{\text{lac}}$, apparent rate constant of lactate elimination (min$^{-1}$); $V_{\text{max}}$, maximum rate of total Glc consumption (mM. min$^{-1}$).  

a 2-tailed, unpaired t-Test: highlighted $p<0.05$; b Pearson correlation: highlighted $\rho>0.8$ ($p<0.05$); c based on the tumor volume (Tv), the average cell density (Cd), and assuming an average GL261 cell radius of 10 µm (Cr, (Roberts et al., 2020)): Tv x Cd x 10$^9$/Cr; d Prevalence of score I (n/Total); e Kendall’s rank correlation (Tau).

Supplementary Table 2. Histopathologic evaluation. Analysis of H&E histologic sections from each tumor and scoring according to phenotypic features of their stromal-vascular fraction.

a predominant; b 0 (absent) or 1 (present); c progression phase; d tumors studied in vivo under acute hypoxia.

Supplementary Table 3. DGE 1H-MRS of GL261 tumors under acute hypoxia.

a $FiO2 = 12\%$

Supplementary Table 4. Differences in voxel vs tumor volumes do not correlate significantly to glucose oxidation or glycolytic rates within each cohort or across pooled cohorts.
SUPPLEMENTARY FIGURES

Supplementary Figure 1. Evaluation of spectral quality and sensitivity of dynamic $^2$H-MRS in vitro. A T2-w RARE image of a homebuilt glass-sphere phantom initially filled with 25 mM D$_2$O solution and connected through an injection line (filled with 500 mM D$_2$O, dead volume) to a syringe loaded with 5 mM 6,6$'$-$^2$H$_2$-glucose in saline (Glc, with DHO natural abundance $\sim$17 mM). B Time-course acquisition of $^2$H-MRS data (pulse-acquire sequence, 67 $^\circ$ flip angle, 1 average, TR=300ms, 200 repetitions, 512 spectral points, and 1749 Hz spectral width) in different conditions: (a) 25 mM D$_2$O; (b) during flush with 500 mM D$_2$O (black arrow); and (c) after injection of 5 mM $^2$H-Glc (red arrow, following the flush in (b)). C Spectral quality (real) obtained in the 3 conditions (a, b, c) and AMARES quantification of deuterated water and glucose peaks (components 1 and 2, respectively; residuals of the fitting in fuchsia). The results show good sensitivity to DHO natural abundance ($\sim$0.03% in water) and physiologic concentrations of glucose (Glc 5 mM), and overall time-course spectral stability.

Supplementary Figure 2. Evaluation of $^2$H-MRS with outer volume suppression in vitro. A 2-compartment phantom was filled with: 20 mM 6,6$'$-$^2$H$_2$-glucose in PBS; and 20 mM $^2$H$_3$-acetate in PBS. $^2$H-MRS acquired with a pulse-acquire sequence, 175ms TR, 256 points, 1749 Hz, 55$^\circ$ flip angle. A No outer volume suppression (OVS). B-C OVS applied outside the phantom, with 6 pulses (10mm slabs), fully covering the acetate compartment (C), or the glucose compartment (C), demonstrating the good performance of volume selection for $^2$H-MRS.

Supplementary Figure 3. Assessment of GL261 tumor perfusion based on DGE T1-MRI in vivo. A Model for transfer Gd-DOTA from the vascular compartment to the intratumor-extracellular space ($K_{\text{trans}}$, min$^{-1}$). B Extended-Tofts for kinetic modelling (GL2 displayed). C Perfusion estimates for all the tumors.

Supplementary Figure 4. Individually tumor scoring of stromal-vascular phenotypes. Distinct morphological features of the stromal-vascular fraction in different GL261 tumors, showing four phenotypes: I, small vessels, complete endothelial cell lining and sparse hemorrhages; II, vasodilation and marked multifocal hemorrhages; III, necrosis of the vascular wall, incomplete endothelial cell lining, vascular leakage, and edematous stroma; and IV, vascular depletion and edematous stroma.

Supplementary Figure 5. Effect of acute hypoxia on glucose metabolism of GL261 in vivo. A Physiologic bench monitoring of n=3 WT mice with the MouseOxPlus® mouse collar sensor, kept under the same conditions of immobilization, anesthesia, and body heating used for MRI (same animal holder). Two conditions tested, with fraction of inspired oxygen ($FiO_2$): 31% and 12%, under normobaric conditions. Time-course measurements of oxygen saturation ($SpO_2$), rectal
temperature, breath and heart rate are displayed, plotted as average values from different animals (left-side). ** Dynamic monitoring of relative brain oxygenation with BOLD T2* MRI, under the two FiO2 conditions in n=1 WT mouse: left-side-top, reference image (first echo under basal condition, 31% FiO2) with ROIs overlaid (red, carotid artery; blue, caudate nucleus); left-side-bottom, respective T2* map (left-side) and r2 goodness-of-fit map (right-side); ΔR2* time-course plots for each ROI (caudate nucleus (top, blue), and carotid artery (bottom, red)) under different FiO2 conditions (1-5 min (grey, basal condition), 31%; 5-7 min (orange, gas adjustment period), from 31 to 12% (§); 7-20min (dark blue, acute hypoxia period), 12%). The results show increased R2* relaxibility under lower oxygenation, consistent with a higher deoxyhemoglobin arterial fraction (red ROI) leading to acute hypoxia in the brain parenchyma (blue ROI). ** C DGE 3H MRS in n=2 GL261 glioma-bearing mice under hypoxia (G8-9, 12% FiO2): left-side, deviation from the Vglx vs. Ki67-LI linearity in the main GL261 cohort under regular anesthesia conditions (G1-7, 31% FiO2: R=0.829, p=0.021); right-side, significantly higher glucose oxidation fraction (Vglx/Vmax, estimate±SE) in GL261 tumors with phenotype IV (n=3) compared to I-II-III (n=4; p=0.026) during regular anesthesia (31% FiO2) not observed in phase IV tumors during acute hypoxia (12% FiO2). Plots: mean±SE. ** p<0.01, *** p<0.001.

Supplementary Figure 6. Effect of averaging DGE 3H-MRS data for MP-PCA denoising. Tumor G4 data displayed as an example. A Resampling the data from 8 to 128 averages (NA) did not impact MP-PCA denoising performance with regards to SNR improvement, but impacted metabolic kinetic profiling and modulation (e.g. Lac at NA=8 vs. 64). B Simulated and real data (ground truth). Three approaches tested with simulated spectra: (first-from-left) adding different noise levels (NL) to the spectra; (second-from-left) changing the total number of spectra in the time-course (equivalent to number of repetitions, NR); or (third-from-left) reproducing the averaging approach used for the real data (forth-from-left) by adapting NL according to NR to reproduce the SNR profile of the original data, in this case from NA= 4 to 256. Best performance highlighted (NA= 64, red arrow).

Highlights:
Glucose fluxes in glycolytic and oxidative pathways detected in vivo by Deuterium Magnetic Resonance Spectroscopy reflect Proliferation in mouse Glioblastoma

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- We performed dynamic glucose enhanced (DGE) $^2$H-MRS in mouse GBM tumors
- Marchenko-Pastur PCA denoising of $^2$H-MRS spectra improved kinetic quantification
- Metabolic kinetics revealed differential glucose pathway fluxes in non-necrotic tumors
- Modulation of glucose metabolism reflected tumor heterogeneity (proliferation)

Credit Author Statement:
Glucose fluxes in glycolytic and oxidative pathways detected in vivo by Deuterium Magnetic Resonance Spectroscopy reflect Proliferation in mouse Glioblastoma

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Conceptualization, RVS and NS; methodology: RVS, RNH, TC, and NS; investigation: RVS, BMC, FFF, and TC; visualization: RVS and TC; funding acquisition: NS and RVS; project administration: RVS; supervision: NS and RVS; writing – original draft: RVS; writing – review & editing: RVS, RNH, BMC, FFF, TC, and NS.