In Vivo 2-Hydroxyglutarate Monitoring With Edited MR Spectroscopy for the Follow-up of IDH -Mutant Diffuse Gliomas

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The *IDASPE* prospective study

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A.L.D.S., M.T., L.C., C.P., B.B., C.D., C.B., A.H. and M.S. provided the cases.

L.N., F.BR., R.V., M.M acquisition, post-processing and analysis of 2HG MRS.

M.G. and J.L. performed the genes analysis.

F. Bi., C.V and JL performed the histological analysis and immunohistochemistry.

C.P. and C.O. analyzed 2 HG tumor tissue concentration.

L.N., D.G., S.L and J.S. assessed radiological responses and tumor volumes variations.

A.L.D.S. and L.N. performed tumor 3D segmentation and tumor volume analysis.

Clinical and histo-molecular data were collected and analyzed by A.L.D.S., GB and M.S.

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Abstract

Background: D-2-hydroxyglutarate (2HG) characterizes *IDH*-mutant gliomas and can be detected and quantified with edited MRS (MEGA-PRESS). Here, we investigated the clinical, radiological, and molecular parameters affecting 2HG levels.

Methods: MEGA-PRESS data were acquired in 71 patients with glioma (24 untreated, 47 treated) on a 3 T system. Eighteen patients were followed during cytotoxic (n=12) or targeted (n=6) therapy. 2HG was measured in tumor samples using gas chromatography coupled to mass spectrometry (GCMS).

Results: MEGA-PRESS detected 2HG with a sensitivity of 95% in untreated patients and 62% in treated patients. Sensitivity depended on tumor volume (> 27 cm^3; p=0.02), voxel coverage (> 75%; p=0.002) and expansive presentation (defined by equal size of T_1 and FLAIR abnormalities, p=0.04). 2HG levels were positively correlated with *IDH*-mutant allelic fraction (p=0.03) and total choline levels (p<0.001) and were higher in *IDH2*-mutant compared to *IDH1*^{R132H}-mutant and non-R132H *IDH1*-mutant patients (p=0.002). In patients receiving IDH inhibitors, 2HG levels decreased within a few days, demonstrating the on-target effect of the drug, but 2HG level decrease did not predict tumor response. Patients receiving cytotoxic treatments showed a slower decrease in 2HG levels, consistent with tumor response and occurring before any tumor volume change on conventional MRI. At progression, 1p/19q codeleted gliomas, but not the non-codeleted, showed detectable *in vivo* 2HG levels, pointing out to different modes of progression characterizing these two entities.

Conclusion: MEGA-PRESS edited MRS allows *in vivo* monitoring of 2-hydroxyglutarate, confirming efficacy of *IDH* inhibition and suggests different patterns of tumor progression in astrocytomas compared to oligodendrogliomas.

Keywords: diffuse gliomas, *IDH1/2* mutations, edited MR spectroscopy, 2-hydroxyglutarate (2HG), noninvasive detection.

Key points:

1) *In vivo* 2HG levels are higher in untreated compared to treated *IDH* mutant patients and 2HG showed a sustained decrease after cytotoxic therapies which was consistent with tumor responses.
2) 2HG detection is affected by radiological features such as tumor volume, voxel coverage and expansive presentation.
3) At progression, only 1p/19q codeleted patients shows detectable levels of *in vivo* 2HG measured by edited MRS.
4) *In vivo* 2HG monitoring provides pharmacodynamic insight on *IDH*-inhibitor action.

Importance of the study:

This study identified several factors affecting the ability to detect 2HG *in vivo* with edited MRS in a large group of patients with *IDH*-mutant gliomas at different stages of the disease, in a real-world setting. Noninvasive, *in vivo* 2HG detection with edited MRS enables to explore *IDH*-mutant glioma biology during conventional or target therapies.

Introduction

Mutations in the isocitrate dehydrogenase (*IDH*) 1 and 2 genes are common in lower grade diffuse gliomas and have important diagnostic, prognostic, and therapeutic implications for patient management[1–3]. Hotspot mutations involving the codon 132 of the enzyme IDH1 and the codon 172 of the enzyme IDH2 result in the intracellular accumulation of D-2-hydroxyglutarate (2HG)[4], an oncometabolite that can be detected non-invasively by *in vivo* magnetic resonance spectroscopy (MRS)[5–8]. However, given its important resonance overlap with other signals, 2HG detection is technically challenging. Several advanced MRS sequences have been proposed to resolve this spectral overlay and to improve the sensitivity and specificity of 2HG detection[9]. Among those, we reported on the 100% positive predictive value of edited MRS using the Mescher–Garwood point-resolved spectroscopy (MEGA-PRESS) sequence for the preoperative diagnosis of the *IDH* status[10]. However, we did not investigate yet the utility of this method for 2HG detection in pretreated patients and for follow-up, which are the main aims of the present study.

The target of edited MRS is the 2HG multiplet at 4.02 ppm, where the signal is slightly lower compared to that at 2.25 ppm detected by non-edited long echo-time (TE) methods, but the overlap with other metabolites is completely removed in the edited spectra. Thus, edited MRS may predict the *IDH*-mutation with higher diagnostic performance compared to more conventional methods.

However, the detectability of 2HG *in vivo* may be hampered by the low sensitivity, proper of all MRS methods, in conjunction with intrinsic low 2HG concentrations related to glioma specificities or treatment effects. The emerging broad clinical impact of 2HG MRS, including treatment planning[11] and response assessment[12–14], urges standardized MRI protocols and precise, personalized clinical indications. In order to improve the interpretability of 2HG MRS results in the context of clinical routine examinations, we investigated the clinical, radiological and molecular factors influencing noninvasive 2HG quantification in a large cohort of untreated and pretreated glioma patients. In addition, in order to explore the effect of different treatments on 2HG levels and

the utility of 2HG MRS to assess response compared to conventional clinical and radiological methods, we evaluated 2HG and tumor volume variations in subgroups of patients with *IDH*-mutant gliomas actively receiving cytotoxic (e.g. radiotherapy (RT) and/or chemotherapy) or targeted therapies.

Study population and Methods

Patient enrollment. Patients were prospectively enrolled at the Pitié-Salpêtrière Hospital (Paris, France) between October 2014 and July 2018. Patients candidate to surgery for a presumed low-grade glioma (cohort 1) or patients followed for a confirmed *IDH*-mutant glioma (cohort 2) were offered to participate to the study.

Inclusion criteria comprised: (1) age ≥ 18 years old, (2) suspected grade 2-3 glioma (cohort 1) or histologically confirmed diffuse *IDH*-mutant grade 2-3 glioma (cohort 2), (3) tumor volume > 6 ml, estimated from T_2/FLAIR images, (4) Karnofsky Performance Status (KPS) > 60, and (5) ability to provide written informed consent and acceptance to sign it. Exclusion criteria comprised: (1) contra-indication to MRI, (2) patient unable to give a written informed consent, and (3) patient already included in group 1.

Enrolled patients were divided into two cohorts based on whether they had (or not) previously received surgery and anti-tumoral treatments:

- Cohort 1: treatment-naïve patients with suspected diffuse glioma on imaging, candidate to receive surgery (biopsy or resection)[10].
- Cohort 2: patients with histologically confirmed *IDH*-mutant diffuse glioma upon biopsy or resection. This cohort included: i) patients who had received, or were actively receiving, anti-tumor treatments, and ii) patients who had measurable 2HG levels by *in vivo* MRS and were about to start a novel line of treatment (either cytotoxic or targeted). The latter underwent

longitudinal MRS evaluations to assess whether *in vivo* 2HG levels could be used to monitor treatment response.

For patients about to start a cytotoxic therapy, MRS imaging was scheduled at baseline and then every 4 months for one year. After one year, when possible, MRS was performed every 4-6 months and at recurrence. Extension of post-treatment MRS was approved by the Sponsor. For patients receiving a targeted therapy, MRS imaging was scheduled at baseline, at 3-7 days after treatment in order to assess the biological efficacy of the drug on 2HG production, and then at 4 weeks and every 2 months for one year.

All patients enrolled in the study underwent the collection of plasma and urine samples at the time of their baseline MRS. Plasma and urines were collected in the hour preceding MRS and stored at -80°C until analysis. For patients in cohort 1, a tumor sample was collected during surgery and snap-frozen at -80°C. We also used a validation cohort of tumor samples for 2HG dosage from the Onconeurotek Tumor bank.

As MRI/MRS does not substitute standard radiological follow-up, patients also had a regular brain MRI within 4 weeks before or after the research MRI/MRS examination, including at least T_1, T_2 FLAIR, T_1 after gadolinium injection, and perfusion MRI when possible.

Standard Protocol Approvals, Registrations, and Patient Consents.

The study was registered on the clinicaltrial.gov database with the number NCT02597335.

Ethical approval was obtained from the institutional review board of *Comité de Protection des Personnes-Paris 6*.

All biological samples and clinico-pathological data were collected upon written informed consent in accordance with the tenets of the Declaration of Helsinki. All subjects provided written informed consent for study participation.

MRI/MRS acquisition protocol. MRI/MRS scans were acquired using a 3 T whole-body system (MAGNETOM Verio, Siemens, Erlangen, Germany) equipped with a 32-channel receive-only head coil. The protocol included a three-dimensional (3D) T_2-weighted FLAIR and a 3D T_1-weighted gradient echo sequence for voxel placement and tissue segmentation, and a diffusion-weighted imaging (DWI) sequence. MRS was acquired using a single-voxel MEGA-PRESS sequence as previously described[10] (T_R = 2 s, T_E = 68 ms, 128 pairs of averages, scan time = 9 min) with editing pulses applied at 1.9 and 7.5 ppm, for the edited and non-edited condition, respectively. Water suppression using VAPOR and outer volume suppression techniques[15] were incorporated prior to MEGA-PRESS acquisition. A non-water suppressed scan was acquired for the quantification of metabolite concentrations. The typical size of the spectroscopic volume of interest (VOI) was 8 cm^3, however, in order to minimize partial volume effects, the VOI was adapted to tumor size keeping a minimum size of 6 cm^3. After tumor segmentation and tumor volumes assessment, we estimated a Voxel Coverage (e.g. the fraction of the VOI filled with tumor).

DWI was acquired using a multiband echo planar sequence (T_R = 1.8 s, T_E = 80 ms, resolution = 2.5 isotropic, multiband factor = 3), 12 diffusion weighting directions at b = 1000 s/mm^2 and one acquisition at b = 0 s/mm^2.

MRS post-processing. Frequency and phase corrections were performed on single spectra based on the total choline signal at 3.2 ppm, using in-house written Matlab routines. The final edited spectra were obtained by subtracting the spectra acquired at the edit-on and edit-off conditions, allowing for the detection of the 2HG signal at 4.02 ppm. Spectral quantification was performed using LCModel (Provencher, 2001), by scaling the metabolite signals to the water reference and assuming a bulk water concentration in tumors of 55.5 mM and a water transverse T_2 relaxation time constant of 150 ms (Madan, 2015). The basis set included the following metabolites: 2HG, cystathionine, γ-aminobutyric acid (GABA), glutamate (Glu), glutamine (Gln), glutathione, *N*-acetylaspartate

(NAA), and *N*-acetylaspartylglutamate (NAAG). Based on our previous findings[10], the Cramer Rao lower bounds (CRLB) threshold for 2HG detection was set to 50%. Edit-off spectra were also quantified using a basis set that included 2HG, ascorbate, aspartate, choline containing compounds (tCho), creatine + phosphocreatine (tCr), GABA, glucose, Gln, Glu, glycine, glutathione, lactate (Lac), *myo*-inositol (mIns), NAA, NAAG, scyllo-inositol and taurine.

Morphological imaging features. Images in DICOM format have been converted to NIfTI using the dcm2nii software. The FLAIR hypersignal was semi-automatically segmented using an active contours and surface algorithm implemented in ITK-SNAP software (version 3.6) under the supervision of the neuroradiologist (LN) who manually corrected and validated tumor segmented images[16,17]. The fraction of the VOI filled with tumor tissue was estimated by overlaying tumor masks from ITK-SNAP with the corresponding VOI using Matlab. Neuroradiological presentation was defined as expansive [if the size of T_1 abnormality was almost equal to the size of FLAIR abnormality] or infiltrative [if the size of T_1 abnormality was much smaller than the size of FLAIR abnormality] according to VASARI feature set v1.1[18]. We assessed the presence of necrotic/cystic areas in accordance with the VASARI feature guide[18]. For patients who had an available contrast MRI acquired for clinical purposes in the 4 weeks before or after baseline MRS, we examined 3D T_1-weighted spin-echo pulse images after gadolinium injection to determine whether any areas of contrast-enhancement were included in the VOI.

Follow-up MRIs from patients of cohort 2 were reviewed and tumor response assessed according to RANO criteria for low-grade or high-grade glioma according to tumor grading at baseline[19,20].

DWI post-processing. Susceptibility-induced off-resonance field was estimated from opposite phase-encoding data using the "topup" tool[21] as implemented in FSL[22] "Eddy" was used to correct for motion and eddy current and EPI distortions (estimated from "topup")[22] . The standard "dtifit"

utility was used to fit the parametric maps of the diffusion tensor. Mean diffusivity and minimal diffusivity were calculated in the spectroscopic voxel.

Immunohistochemical and molecular studies on tumor specimens. Tumor diagnoses were established by two board-certified neuropathologists (FBi, CV), according to the 2021 WHO classification of Tumors of the Central Nervous System[1]. Immunohistochemistry was performed on 4-µm-thick formalin-fixed paraffin embedded sections using an automated system (Benchmark XT, Ventana Medical System Inc, Tucson AZ, USA) for the assessment of *IDH1*^{R132H} (Dianova, H09), p53 (DAKO, DO.7) and ATRX (SIGMA, polyclonal) expression. All tumors negative for *IDH1*^{R132H} on immunohistochemistry were assessed for *IDH1* and *IDH2* mutations by DNA sequencing. Tumor DNA was extracted from snap-frozen tissue (QIAamp DNA Mini Kit, Qiagen) or from formalin-fixed paraffin embedded sections (GeneJET FFPE DNA purification kit, Thermo Scientific) as per manufacturers' instructions. The mutational status of *IDH1* and *IDH2* was determined using the Sanger technique and previously reported primers[23] or by Next-Generation Sequencing (NGS). Copy number variations were assessed by CGH array[23] or NGS. For patients in cohort 1, we estimated *IDH*-mutant variant allelic fraction in the surgical specimen from NGS data (number of mutant reads over total number of reads) or from the visual assessment of the chromatograms from Sanger sequencing.

Immunohistochemical measurement of tumor cellularity. The tissue section corresponding to the formalin fixed and paraffin embedded block with the highest tumor cellularity was selected by central review of the standard staining by a senior neuropathologist (FBi). Three micrometer-thick tissue sections of the selected blocks were processed by an automated stainer Ultra (Ventana, Roche, Basel, Switzerland) using rabbit monoclonal anti-SOX2 (SP76; 1/300; Sigma-Aldrich) as primary antibody, diaminobenzidine as chromogen and hemalun as blue nuclear counterstaining. Sections were scanned with Axioscan (Zeiss) and three ROIs (Regions of Interest) (440 µm^2) were

captured with Zen software (Zeiss). Three zones were chosen: one with the highest intensity, one with the lowest and one with medium intensity staining. ROIs were then analyzed with ImageJ software to quantify the SOX2 positive surface, the number of SOX2 positive nuclei and the number of SOX2 negative nuclei. An average of the three zones was calculated.

2HG concentrations in tumor tissue, plasma, and urine. Snap-frozen tumor samples from patients in cohort 1 and from an independent group of patients with diffuse gliomas identified in the OncoNeuroTek database (Pitié-Salpêtrière, Paris) were analyzed by the tandem gas chromatography-mass spectrometry (GC-MS) to assess 2HG concentrations, as previously described[10]. The control group was deliberately enriched with tumors with uncommon *IDH1* and *IDH2* mutations to allow subgroup analyses. 2HG concentrations in plasma and urine samples were assessed by GC-MS using the same methods described for tumor tissues[10].

Statistical analyses. Differences in the distribution of categorical variables between groups were assessed using Fisher's exact test. Differences between groups with respect to quantitative variables were tested with Wilcoxon's test. Multidimensional association tables were used to explore associations between variables, estimated by the Pearson correlation coefficient. Overall survival (OS) was defined as the time between tumor diagnosis and death (or last follow-up). Survival curves were calculated using the Kaplan-Meier method. Differences between survival curves were assessed using the Log-Rank test. The Cox model was applied to evaluate the effect of quantitative variables on OS and for multivariate survival analyses. All statistical tests were 2-sided at 0.05 significance level. Statistical analyses were performed using R.

Data availability. Additional data, including study protocol, and data not provided in the article because of space limitations may be shared (anonymized) at the request of any qualified investigator.

Results

Cohort description. We prospectively enrolled and studied by edited MRS 71 consecutive patients (38 males, 33 females, median age 44 y (range:21-67)), including patients with suspected diffuse glioma on imaging, before surgery and *naive* of any anti-cancer therapy (cohort 1 corresponding to 20 *IDH* mutant (9 grade 2, 10 grade 3, one grade 4) and 4 *IDH*-wild-type (one grade 1, 2 grade 2, one grade 4))[10], and 47 patients with a histologically confirmed diagnosis of *IDH*-mutant glioma (cohort 2). Among the latter, 18 patients were enrolled following initial surgery and before any treatment, 26 patients were enrolled after having received (or while actively receiving) anti-tumoral treatments, while the information was missing for three patients. Eighteen patients from cohort 2, who had detectable 2HG levels measured by MRS (2HG-MRS) and were about to start conventional cytotoxic treatments (n=12) or IDH inhibitors (n=6), underwent longitudinal MRS assessments for 2HG monitoring.

Clinical, radiological, and molecular factors affecting 2HG detection by in vivo edited MRS. 2HG was quantified in 53 out of 67 patients with *IDH*-mutant tumors and in none of the 4 patients with *IDH*-wild-type tumors, corresponding to a sensitivity of 79% and a specificity of 100%. **Table 1** compares the main clinical, radiological, and histo-molecular features of patients with *IDH*-mutant gliomas with detectable vs. undetectable 2HG-MRS.

2HG detection is affected by previous and ongoing treatments. 2HG was less commonly detected in patients with history of surgery ($p=0.04$) and cytotoxic treatments ($p<0.01$) (**Table 1**), with 2HG detection rate decreasing from 95% in preoperative patients to 62% in patients having received surgery and cytotoxic treatments.

2HG-MRS levels were lower in patients who had undergone surgery compared to patients with naïve *IDH*-mutant gliomas (median: 1.5 vs. 2.5 mM, p=0.021) (**Figure 1, panel A**), but this difference was no longer significant when the 2HG levels were normalized by the percentage of tumor included in the voxel (median: 2.3 vs 2.4, p = 0.3), suggesting that 2HG is affected by tissue content in the voxel.

2HG levels were also lower in patients who had received cytotoxic treatments compared to patients with no past anti-tumoral therapy (median: 1.1 vs. 2.3 mM, p=0.026) (**Figure 1, panel B**). Patients under chemotherapy at the time of MRS had lower 2HG-MRS compared to patients who were not receiving treatment (median: 0.2 vs. 2.3 mM, p=0.012) (**Figure 1, panel C**).

2HG detection is affected by tumor cell density and IDH mutation type. We investigated whether the decreased ability to detect 2HG in pretreated patients could be attributed to the reduction of viable tumor cells associated with anti-tumoral treatment. As expected, 2HG detection was associated with better voxel coverage by the tumor target (> 75%; p<0.001) and expansive presentation (p=0.04) (**Table 1; Figure 1, panel D**). Contrast enhancement within the voxel (p=1), mean diffusivity (p=0.9), and relative cerebral blood volume (rCBV) (p=1) were not associated with 2HG detection. We found a weak negative correlation between 2HG-MRS and minimal diffusivity values (r=-0.3, p=0.045), which were suggested to reflect cell density (**e-Figure 1, panel A**)[24]. 2HG-MRS directly correlated with tCho levels (r=0.58, p<0.001) (**e-Figure 1, panel B**), reflecting cell membrane turnover and cellularity, and inversely correlated with mIns levels (r=-0.29, p=0.002) (**e-Figure 1, panel C**), reflecting gliosis[25,26], typically elevated in gliomatosis cerebri characterized by a low density infiltration by glioma cells[27,28]. These results are in line with the hypothesis that the density of tumor cells within the voxel is likely to be a determining factor of 2HG detection.

As our findings suggested that the proportion of tumor cells in the voxel might affect 2HG detection, we assessed whether 2HG-MRS was correlated with the proportion of tumor cells in the surgical sample of patients of cohort 1 (median interval between MRI/MRS and surgery was 1

day)[10]. 2HG levels measured by MRS (r=0.50, p=0.033) (**e-Figure 1, panel D**) and 2HG concentration in tumor tissue measured by GC-MS (r=0.53, p=0.023) (**e-Figure 1, panel E**) were both correlated with the proportion of *IDH*-mutant reads, which is a surrogate for tumor cell fraction, at least at the early stage of the disease as *IDH* mutation is a truncal and early event.

We then investigated whether 2HG tumor levels were associated with the molecular profile. Patients with *IDH2* mutations had higher 2HG levels than non-R132H *IDH1* mutations, while patients with $IDH1^{R132H}$ tended to have an intermediate level (mean: 6.20 vs. 2.27 vs. 1.11 mM, p<0.01) (**e-Figure 2**). No association was found with 1p/19q codeletion (data not shown).

2HG-MRS correlates with 2HG concentrations in plasma. We evaluated whether measuring 2HG in easily accessible body fluids, such as plasma or urine, could reliably monitor 2HG levels in tumor tissue. 2HG concentrations in plasma correlated with 2HG levels measured by MRS (r=0.32, p=0.024) and with total 2HG contained in the tumor estimated by 2HG concentration x tumor volume (r=0.37, p=0.01) (**e-Figure 1, panel F**), while 2HG concentrations in urine did not correlate either with 2HG levels estimated by MRS (r=-0.29, p=0.57) or with 2HG concentrations measured in tumor tissue by GC-MS (p=0.72).

2HG levels at progression are lower in 1p/19q non-codeleted compared to 1p/19q codeleted IDH-mutant gliomas. As 2HG was more frequently undetectable in pretreated patients than in those examined before surgery, we sought to determine whether this could be related to a more advanced stage of disease, considering that even though *IDH* mutations is an truncal event, recent work showed that it may be lost at progression as tumor acquired tertiary alterations[29,30]. A substantial proportion (9/25, 36%) of patients had no detectable 2HG-MRS at progression (**Figure 2, panel A**). Strikingly, this was mostly the case for *IDH*-mutant 1p/19q non-codeleted gliomas who, at progression, showed lower median levels of 2HG compared to codeleted gliomas (median: 0.6. vs. 3.7 mM, p=0.023) (**Figure 2, panels B, C, D**). Similarly, while 1p/19q non-codeleted gliomas displayed lower 2HG levels the more time elapsed from surgery, 1p/19q codeleted gliomas

maintained elevated 2HG levels over time (**e-Figure 3, panels A and B**) suggesting differences in the longitudinal evolution and mechanisms of progression between the two subtypes of *IDH* mutant gliomas.

Since we found that lower 2HG levels were associated with a more advanced stage of the disease, we wanted to assess whether 2HG levels *per se* predicted further survival (measured from the time of the MRI to death). Instead, the association of 2HG levels with further survival (univariate analysis, p=0.006) was merely explained by the inverse correlation between 2HG levels and stage of the disease (defined as the time elapsed from initial diagnosis to MRS). Early stage of the disease (p=0.001), lower grade (p=0.033) and 1p/19q codeletion (p=0.049) were the only predictors of further survival. Furthermore, 2HG measured in cohort 1 (e.g. at diagnosis) either with MRS or GC-MS was not correlated with survival (HR=1.12 (0.54-2.33), p=0.75) or PFS (HR=1.005 (0.67-1.5), p=0.98).

2HG-MRS rapid decrease after treatment with IDH inhibitors demonstrates pharmacodynamic effect. Six patients (4 with non codeleted glioma, 2 with codeleted glioma) were treated at recurrence after conventional treatment with IDH1 (AG120, n=4) or IDH2 (AG221, n=2) inhibitors, and underwent longitudinal MRS assessments during treatment. In all patients, 2HG levels rapidly and dramatically decreased and became undetectable within a few days from the beginning of the treatment. 2HG levels rebounded very quickly after permanent or temporary treatment discontinuation **(patients 1, 5, 6, Figure 3).** 2HG decrease did not predict tumor volume response **(patients 2 and 4, Figure 3**), while a progressive increase of 2HG and a mild volume progression during treatment was observed in patient 3 suggesting resistance mechanism associated with maintained 2HG production possibly due to suboptimal enzyme inhibition.

2HG-MRS decrease anticipates tumor volume response after cytotoxic treatments. Twelve patients in cohort 2 underwent longitudinal 2HG assessment by MRS while receiving cytotoxic treatments (temozolomide (n=2), PCV (n=1), PCV-RT (n=5), RT-PCV (n=4). Individual 2HG and tumor volume variations curves are shown in **e-Figure 4**. On average 2HG-MRS decrease was observed four months after starting a treatment (**Figure 4, panel A**), preceded tumor volume (**Figure 4, panel B)** and tCho longitudinal changes (**Figure 4, panel C**) and continued over the twelve months of follow-up (**Figure 4, panel A**). 2HG decrease was similar in patients with 1p/19q codeleted gliomas and 1p/19q non-codeleted gliomas (**Figure 5, panels A and B**) or after the sequence radiotherapy followed by chemotherapy and chemotherapy followed by radiotherapy **(Figure 5, panel C and D; Figure 6; e-Figure 4**). Interestingly, three patients (two 1p/19q codeleted and one 1p/19q non codeleted) experienced tumor progression during follow-up. In line with our former finding, only the 1p/19q codeleted patients had a detectable concentration of 2HG at progression (**Figure 5, panels A and B**).

Discussion

We recently reported that edited MRS performed with the MEGA-PRESS sequence is a highly sensitive and specific method for the preoperative prediction of the *IDH* status in patients with diffuse gliomas[10]. However, while showing a sensitivity of 95% in untreated patients, the sensitivity of MEGA-PRESS in pretreated patients dropped to 62% (using a CRLB cutoff of 50%). As confirmed by our multidimensional analysis, the decrease in tumor volume, voxel coverage, and viable tumor cells that occur after tumor debulking and cytotoxic treatments probably concur to the lower rate of 2HG detection observed in pretreated patients. These results are in line with previous studies that identified tumor volume[14,31] and cellularity[12,31] as important determinants for 2HG detection by MRS. Longitudinal monitoring by MRS allowed to capture a rapid decrease of

2HG in patients receiving IDH inhibitors, within days after the beginning of the treatment. This result is in line with a previous study conducted on a small cohort of patients receiving the IDH305 inhibitor[14] and demonstrates that the drug efficiently inhibits its target. However, the prolonged monitoring performed in the present study showed that efficient IDH inhibition, detected by decreased 2HG levels, does not necessarily translate into tumor response. This observation confirms that 2HG-MRS is a unique tool for evaluation of efficient IDH inhibition and provides clues into the mechanism by which tumors may resist to IDH inhibitors. However, our preliminary data showed that 2HG-MRS monitoring failed to predict correctly tumor progression in patients receiving IDH inhibitors (see patients 2 and 4), with the need to rely on conventional imaging for response assessment, as our data suggest two different patterns of progression, one dependent on 2HG, and the other one 2HG-independent.

Conversely, patients receiving cytotoxic treatments showed a gradual reduction of 2HG-MRS over 12 months (see figure 5) that anticipated tumor volume variations. In line with previous reports[12,13,31], this observation confirms that 2HG-MRS might be effectively used for early response assessment in clinical trials for *IDH*-mutant gliomas.

Importantly, we observed that oligodendrogliomas (1p/19q codeleted) and astrocytomas (1p/19q non-codeleted) differed in 2HG levels at progression or advanced stages of the disease. While oligodendrogliomas showed high levels of 2HG at progression, astrocytomas more commonly showed low or undetectable levels of 2HG, suggesting that astrocytomas might not necessarily rely on 2HG for tumor progression. These preliminary findings, if confirmed in a larger patient population, may have crucial clinical implications for prediction of malignant progression and patient outcome. Indeed, *IDH* mutations may be lost at recurrence as additional "tertiary" oncogenic alterations appear, leading to a more aggressive tumor (grade 3 or 4 astrocytomas), while the epigenetic changes induced by 2HG do not regress after the loss of the *IDH* mutation or 2HG removal[32]. The loss of DNA methylation in recurrent *IDH*-mutant gliomas (G-CIMP-low) has been associated with the acquisition of genetic alterations in the RB pathway (e.g., *CDKN2A/B*

homozygous deletion and/or *CDK4* amplification) and a more aggressive phenotype[33,34], and this primarily occurs in non-codeleted gliomas, while codeleted gliomas do not change dramatically in terms of epigenetic profile[33]. These last observations further stress the great utility of 2HG MRS in exploratory researches on biological tumor variations along glioma disease evolution.

We also observed that 2HG levels correlated with the type of *IDH* mutation, higher in *IDH2* mutated, and lower in *IDH1* non R132H mutated tumors compared to $IDH1^{R132H}$. This observation is consistent with previous reports showing that 2HG levels are higher in *IDH2*- compared to *IDH1*-mutant gliomas[35,36]. Notably, the different *IDH* mutations are associated with different glioma types, *IDH2* mutations being more frequent in 1p/19q codeleted oligodendrogliomas[37], and non-R132H *IDH1* mutations being commonly associated with astrocytomas and, especially, *IDH*-mutant intrinsic pontine gliomas[38]. These preferences may reflect different enzymatic properties and be related to different histological and microenvironmental context. Indeed, each mutation of *IDH1* and *IDH2* results in a different isoform with different enzymological characteristics (Km and Vmax), 2HG production depending on both intracellular alpha-ketoglutarate concentration and the type of mutation[39].

In conclusion, we showed that 2HG measured by edited MRS is a useful and promising noninvasive tool not only for the diagnosis, but also for the follow-up of *IDH*-mutant diffuse gliomas, in contrast to plasmatic 2HG concentrations, whose accuracy was too low to make this measure a reliable surrogate marker of the *IDH* mutation. In addition, the MRS data shown here suggest two different patterns of tumor progression in 1p/19q codeleted, versus non-codeleted *IDH*-mutant gliomas, with important therapeutic implications, as IDH inhibition is an inappropriate strategy in 2HG-independent tumor progression.

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Table 1. Clinical, histomolecular, and radiological features in *IDH*-mutant patients with detectable (2HG+) and undetectable (2HG-) 2HG by *in vivo* edited MRS.

	2HG+ (n=53)	**2HG- (n=14)**	**p**
[2HG] (mM), median (range)	2.66 (0.61-9.70)	0.00 (0.00-0.90)	**<0.0001**
Demographic features:			
Age (years), median (range)	44 (24-67)	42 (30-64)	0.93
Female/male	27/26	5/9	0.38
Histomolecular features:			
IDH mutation, n (%)			
IDH1 R132H	46/53 (86%)	14/14 (100%)	
IDH1 R132 minor mutations	4/53 (8%)	0/14 (0%)	0.62
IDH2	3/53 (6%)	0/14 (0%)	
1p/19q codeletion, n (%)	22/50 (44%)	3/14 (21%)	0.21
Histological grade, n (%)			
2	25/53 (47%)	5/14 (36%)	
3	26/53 (49%)	6/14 (43%)	0.16
4	2/53 (4%)	3/14 (21%)	
Radiological features:			
Tumor volume (cm^3), median (range)	57.6 (10.7.-268.0)	29.0 (3.3-186.6)	0.09
Voxel coverage (%), median (range)	91 (20-100)	75 (1-93)	**<0.001**
Expansive presentation, n (%)	43/52 (83%)	8/14 (57%)	**0.04**
Contrast enhancement, n (%)	17/27 (63%)	5/7 (71%)	1
Previous treatments:			
Past surgery, n (%)	33/52 (63%)	13/14 (93%)	**0.04**
Past CHT and/or RT, n (%)	16/50 (32%)	10/14 (71%)	**<0.01**
Past RT, n (%)	10/50 (20%)	10/14 (71%)	**<0.001**
Past CHT, n (%)	13/50 (26%)	9/14 (64%)	**<0.01**

Legend to Table 1. CHT=chemotherapy; 2HG=2-hydroxyglutarate; MRS=magnetic resonance spectroscopy; RT=radiotherapy.

Figure legends

Figure 1. *In vivo* 2HG levels measured in different patient groups and parameters affecting 2HG quantification. 2HG levels are reported depending on the presence or absence of A) previous surgery (median: 1.5 vs. 2.5 mM, p = 0.021); B) previous cytotoxic treatment (median: 1.1 vs. 2.3 mM, p = 0.026); and C) ongoing chemotherapy (median: 0.2 vs. 2.3 mM, p = 0.012). Horizontal lines indicate the median and the interquartile range. D) Correlation matrix of *in vivo* 2HG levels and main clinical, histo-molecular and radiological parameters obtained in the whole data set of 67 *IDH* mutant glioma patients. The color scale bar indicates the correlation coefficients. Significant correlations ($p < 0.05$) are shown.

Figure 2. *In vivo* 2HG levels measured at different time-points. A) *In vivo* 2HG levels measured before surgery (T1, 2.7 mM), after surgery (T2, 2.2 mM), during or after a first-line treatment (T3, 1.2 mM), after first or second progression (T4: P1 P2, 1.0 mM), and after third or subsequent progression (T5: P3 P4, 4.9 mM). B) *In vivo* 2HG levels at progression in patients with *IDH*-mutant, 1p/19q codeleted vs. *IDH*-mutant 1p/19q non-codeleted gliomas (median: 3.7 vs. 0.6 mM, p=0.023). (C, D) *In vivo* 2HG in *IDH*-mutant, 1p/19q codeleted or 1p/19q non-codeleted gliomas at different time-points of evolution. Horizontal lines indicate the median and the interquartile range.

Figure 3. *In vivo* 2HG level (upper row, asterisks) and tumor volume (bottom row, triangles) longitudinal changes in six patients receiving IDH inhibitors. Treatment period corresponds to the grey shaded area. Undetectable levels of 2HG (CRLB>50%) were set to zero. Clinical and radiological progression according the RANO criteria is indicated by the black arrow. Tumor volumes are reported as percent with respect to baseline.

Figure 4. *In vivo* 2HG levels in patients receiving cytotoxic treatments, compared to tumor volumes and tCho levels. (A) *In vivo* 2HG, (B) tumor volume (percent with respect to baseline),

and (C) *in vivo* tCho longitudinal changes during follow-up in the whole group. The number of patients included at different time points is reported upon error bars.

Figure 5. *In vivo* 2HG levels in patients receiving cytotoxic treatments. 2HG levels during follow-up in (A) *IDH*-mutant 1p/19q codeleted and (B) 1p/19q non-codeleted gliomas, in patients receiving (C) radiotherapy followed by PCV or (D) PCV followed by radiotherapy. The number of patients included at different time points is reported upon error bars.

Figure 6. Evolution of a 1p/19q codeleted anaplastic oligodendroglioma. Upper part: the FLAIR images of the patient treated with surgery followed by RT-PCV show a progressive reduction of the tumor volume. MEGA-PRESS spectra (lower part for each time point) show detectable 2HG before RT (2.7 mM) and a progressive 2HG decrease during treatment (1.0 mM after RT, undetectable level during PCV at 8 and 12 months) and follow-up without treatment.

Supplementary data

e-Figure 1. Correlation of *in vivo* 2HG levels with A) minimal diffusivity (p=0.045); B) tCho levels (p<0.001); C) mIns levels (p=0.002); D) *IDH*-mutant allelic fraction (p=0.033). E) Correlation between 2HG levels in tumor tissue measured by GC-MS and *IDH*-mutant allelic fraction (p=0.023). F) Correlation between total 2HG contained in the tumor (estimated by *in vivo* 2HG concentration x Tumor volume) and 2HG concentrations measured in plasma by GC-MS (p=0.01).

e-Figure 2. *In vivo* 2HG levels and 2HG concentrations in tumor tissue according to the type of *IDH* mutation. IDH-wildtype gliomas vs. non-R132H-*IDH1* mutations vs. *IDH1*^{R132H} mutations vs.

IDH2 mutations. A) 0.00 vs. 1.11 vs. 2.27 vs. 6.20, p<0.001. B) 0.94 vs. 67.23 vs. 111.04 vs. 110.78 mM, p<0.001.

e-Figure 3. *In vivo* 2HG levels measured at different time-points, according to the time elapsed from initial surgery in (A) 1p/19q codeleted and (B) 1p/19q non codeleted *IDH*-mutant gliomas.

e-Figure 4: Individual *in vivo* 2HG levels (A, D), tumor volume (B, E), tCho (C, F) longitudinal changes in glioma treated with RT-PCV (A,B,C) or PCV-RT (D, E, F). Each line corresponds to a patient. Arrows indicate the timeline of subsequent treatments.