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MR-Spectroscopy and Survival in Mice with High Grade Glioma Undergoing Unrestricted Ketogenic Diet

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ABSTRACT

Glioblastoma multiforme (GBM) is considered the most malignant form of primary brain tumor. Despite multimodal treatment, prognosis remains poor. Ketogenic diet (KD) has been suggested for the treatment of GBM. In this study, the syngenic, orthotopic GL261 mouse glioma model was used to evaluate the effects of KD on the metabolic responses of the tumor using 7T magnetic resonance imaging/spectroscopy. GL261 cells were injected into the caudate nucleus of mice. Following implantation, animals were fed with standard chow or underwent a KD. 18 days after initiating the diet, mice fed with KD displayed significantly higher plasmatic levels of ketone bodies and survived longer than those fed with the standard diet. Decreased concentrations of gamma-aminobutyric acid, N-Acetyl-Aspartate and N-acetylaspartylglutamate were found in tumor tissue after 9 days into the KD, while a huge increase in beta-hydroxybutyrate (bHB) was detected in tumor tissue as compared to normal brain. The accumulation of bHB in the tumor tissue in mice undergoing the KD, may suggest either elevated uptake/release of bHB by tumor cells, or the inability of tumor cells in this context to use it for mitochondrial metabolism.

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Introduction

Gliomas are a group of central nervous system tumors of glial cell origin, accounting for at least 50% of all primary intrinsic brain tumors covering various primary tumor types with histological features similar to glia (astrocytoma, oligodendroglioma and ependymoma) [1].

Among gliomas, glioblastoma multiforme (GBM), a grade IV glioma classified by the World Health Organization (WHO), is considered the most malignant and invasive subtype with a median survival rate of 14.6 mo, [2]. GBM is characterized by histopathologic features of cellular atypia, severe necrosis and a high rate of angiogenesis. Despite multimodal treatment consisting of surgical resection followed by concomitant chemotherapy and focal radiotherapy, there is no cure for this deadly disease [3,4].

Anti-angiogenic treatments such as bevacizumab have shown some promise in the context of recurrent disease [5], but have yielded no effect on overall survival when combined with first-line treatments [6,7].

Nevertheless, there is a growing interest in the metabolic management in brain cancer [8–11]; studies in different glioma mouse models have demonstrated that a calorically-restricted high-fat, low-carbohydrate diet, often referred to as ketogenic diet (KD), may be effective in decreasing vascularity, increasing programmed cell death and diminishing levels of insulin-like growth factor [12–14].

Moroever, in human diseases, KD has long been used in specific groups of chronic pediatric and adult patients with epilepsy to reduce frequency and severity of seizures [15–17].

The rationale for following a KD in the treatment of brain tumors is based on the assumption that brain tumor cells, differently from healthy neural tissue, are dependent on glucose for their cellular growth and

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survival and are unable to rely on ketone bodies for their energetic needs [18]. According to these evidences, GBM cancer cells would be unable to use ketones and thus would remain largely dependent on glucose as their metabolic substrate [19–21]. Therefore glucose depletion through the (calorie-restricted)-KD would deprive glioma cells of their critical energy supply preventing tumoral cell growth. Furthermore, it has been shown that even non caloric-restricted KD may prolong survival in experimental glioma [22].

Following these observations, case reports [10] as well as phase I–II clinical trials have explored the feasibility and possible effectiveness of a KD, either unrestricted or calorically-restricted, in high grade gliomas yielding some preliminary evidence for its tolerability and efficacy [23,24].

However, experimental data supporting the inability of malignant glioma cells to use ketone bodies as an energetic substrate remain conflicting [25], raising the need for continuing research in preclinical (*in vitro* and *in vivo* animal models) and clinical settings. In this context, MRI-spectroscopy provides the possibility to monitor i*n vivo*the putative metabolic shifts in tumor and healthy brain tissue in high grade gliomas of animals undergoing KD.

In this paper we provide further data in a well-established, syngenic, intracranial orthotopic mouse high grade glioma model that displays many similarities to human gliomas, assessing the effects of unrestricted KD on clinical MRI-spectroscopy and metabolic responses of the tumor.

Materials and Methods

Health and Safety

All mandatory health and safety procedures have been complied during the course of the study, both *in vitro* and *in vivo experimental settings*

Cell Cultures

The malignant glioma cells syngeneic with C57BL/6 mice used in our study were kindly provided by Dr. G Safrany [26]. After cell thawing, a total of 5×10^5 GL261 cells at passage 16 were plated and cultured in 75 cm^2 flasks (Corning) using DMEM (Dulbecco's Modified Eagle's Medium, Gibco) supplemented with 10% of foetal bovine serum (South America FBS, Gibco) and antibiotics (penicillin/streptomycin (PS) 1%, Gibco). The cells were maintained in culture for two passages 80% confluency and then used for the experiments.

Table 1. abbreviation for MRI analyzed compounds.

bHb	β-hydroxybutyrate
Cr	Creatinine
Cr + PCr	Creatine + Phosphocreatine
CrCH2	Creatine methylene
GABA	gamma-aminobutyric acid
Gln	Glutamine
Glyc	glycerol
GPC	Glycerol phosphorylcholine
Lip13a	Lipid 13a
NAA	N Acetyl Aspartate
NAAG	N-acetylaspartylglutamate
PCr	Phosphocreatinine

Brain Tumor Model

10-week old female mice (C57BL/6N) were maintained under controlled conditions of temperature (22–24 °C), humidity (45–65%) and light (12 h light-dark cycle), with access to food and water *ad libitum*. Mice were housed in standard plastic cages (6/cage) bedded with wood shavings with the addition of some shredded filter paper for nest building. Animals were allowed to acclimate for at least one week before beginning the experiments.

Subconfluent GL261 cells were harvested by trypsinization, washed with cold PBS and resuspended in DMEM at a concentration of 1000 cell/μL. Subconfluent GL261 cells were implanted in ten week-old females C57BL/6N mice (Charles River Laboratory, Calco-Lecco Italy).

Mice were injected with 10^5 GL261 cells into the nucleus caudatum using the stereotactic coordinates 0.7 mm posterior, 3 mm left lateral, and 3 mm deep with respect to the bregma. Capacity to eat and move were monitored daily and animals were weighed three times a week. Three animals per group regularly underwent MRI at 8, 16, 24, 32 and 40 day after glioma cell injection. Glucose and ketones levels in the serum were measured at day 0, 18, 30 and prior to euthanization using tail blood after puncturing the tail with a 25 gauge needle. Glucose and ketone levels were measured using a StatStrip glucose/ketone Meter System (Nova Biomedical, Waltham, USA).

All animal procedures were conducted following standard veterinary practice in accordance with the European Communities Council directives (2010/63/UE). Project Number LA-01-14.

Ketogenic Diet

Following GL261 implantation, animals were fed standard rodent chow (Charles River) for five days then were randomly assigned to either standard chow diet (GL261-CTR) or KD (GL261-KD) both with

access to water and food (*ad libitum*) until the animals were sacrificed.

Standard chow diet was composed of 18.5% protein, 3% fat, 6% fiber and 46.2% carbohydrate (cat number 4RF21, Mucedola s.r.l.; see Supplementary material Table 1A for detailed content of standard chow). The low carbohydrate ketogenic diet was purchased from sniff Spezialdiäten GmbH (catalogue number 15149, Germany). This diet provides calories from 79.2% crude fat from which metabolized energy derives for 94% from fat and 6% from crude proteins and carbohydrates (Supplementary material, Table1A and B). The KD was replenished daily with with no calories restriction on a petri dish within the cage. Food intake was calculated twice weekly by subtracting the weight of the leftover food from the weight of the total amount of food provided and dividing by the number of mice.

Magnetic Resonance Imaging (MRI) and Magnetic Resonance Spectroscopy (MRS)

The *in vivo* MRI and MRS experiments were performed by a horizontal-bore preclinical scanner (BioSpec 70/30 USR, Bruker, Ettlingen, Germany). The system has a magnetic field strength of 7 T and a 30 cm bore diameter. The scanner was equipped with an actively shielded gradient system with integrated shims set up to 2nd order. The maximum gradient amplitude is 440 mT/m. All acquisitions were carried out using a cross coil configuration. A 72 mm linear birdcage RF coil was used for radiofrequency excitation and a mouse brain surface coil was used for signal reception.

All procedures were performed under spontaneous ventilation. Animals were initially anesthetized with Isoflurane 4–5% in a 20% O_2/80% vol:vol air mixture (induction chamber) and maintained with Isoflurane 1.5–2% in a 20% O_2/80% vol:vol air mixture (flow rate 0.8 liter/min). Animal health conditions and respiratory rate, hence the anesthesia depth, was monitored during MRI procedures by a pneumatic sensor (Small Animals Instruments Inc., NY, USA). Animals' temperature was maintained at 36–37 °C by means of a warm-water circuit integrated into the animal bed.

A set of three orthogonal T2-weighted images were acquired as anatomical reference and for spectroscopy voxel placement. Rapid Acquisition with Refocused Echoes (RARE) sequences were performed with the following parameters: TR = 3000 ms, TE = 13.5 ms, FOV = 2.2 × 2.2 cm^2, matrix size = 256 × 256, slice thickness = 0.8 mm, RARE factor = 8, number of averages NA = 8, acquisition time 9 min 30 s.

Proton spectroscopy was carried out by a PRESS (Point RESolvedSpectroscopy) sequence from a single voxel located inside the tumor (or, before detecting solid tumor, at the injection site) and in the contralateral hemisphere.

PRESS sequence parameters were the following: TR = 4776 ms, TE = 9.3 ms, Voxel size 2.5 × 2.2 × 1 mm, Bandwidth 4006 Hz, Spectral resolution 0.24 Hz/point, Number of averages NA = 600, Acquisition Time 48 mins,.

Before acquisition, first and second order localized shimming procedure was performed.

Optimized water suppression pulses were employed.

Spectral data were analyzed with LCModel software (Version 6.3-1J by Stephen Provencher) for metabolites absolute quantification [27,28].

Statistical Analysis

GraphPad Prism 4.0 software (Statcon, Witzenhausen, Germany) was used for statistical analyses. Differences in survival were evaluated by Kaplan Mayer curves and log-rank analysis. Statistical significance between groups was assessed by t-test (unpaired, two-tailed) or one-way ANOVA followed by specific post hoc tests. Statistical significance was established at p values <0.05.

Results

Growth of Glioma in Mouse Brain after Intracranial Injection of 10^5 GL261 Cells

After injection of GL261 cells in mouse brain, 90% of mice presented implantation and growth of brain tumors. In Figure 1a, tumor growth over the course of a few weeks is reported: Between day 5 and 7, glioma cells started to become visible and grew substantially occupying most of the left hemisphere over the course of a few weeks (Figure 1a).

Effects of KD on Cellular Survival, Blood Glucose and Ketone Bodies Measurements

Mice fed KD *ad libitum* displayed a slightly, but statistically significant longer survival rate compared to the control group (Figure 1b). The impact of the diet on body weight was also significant starting at day 18 (Figure 1c).

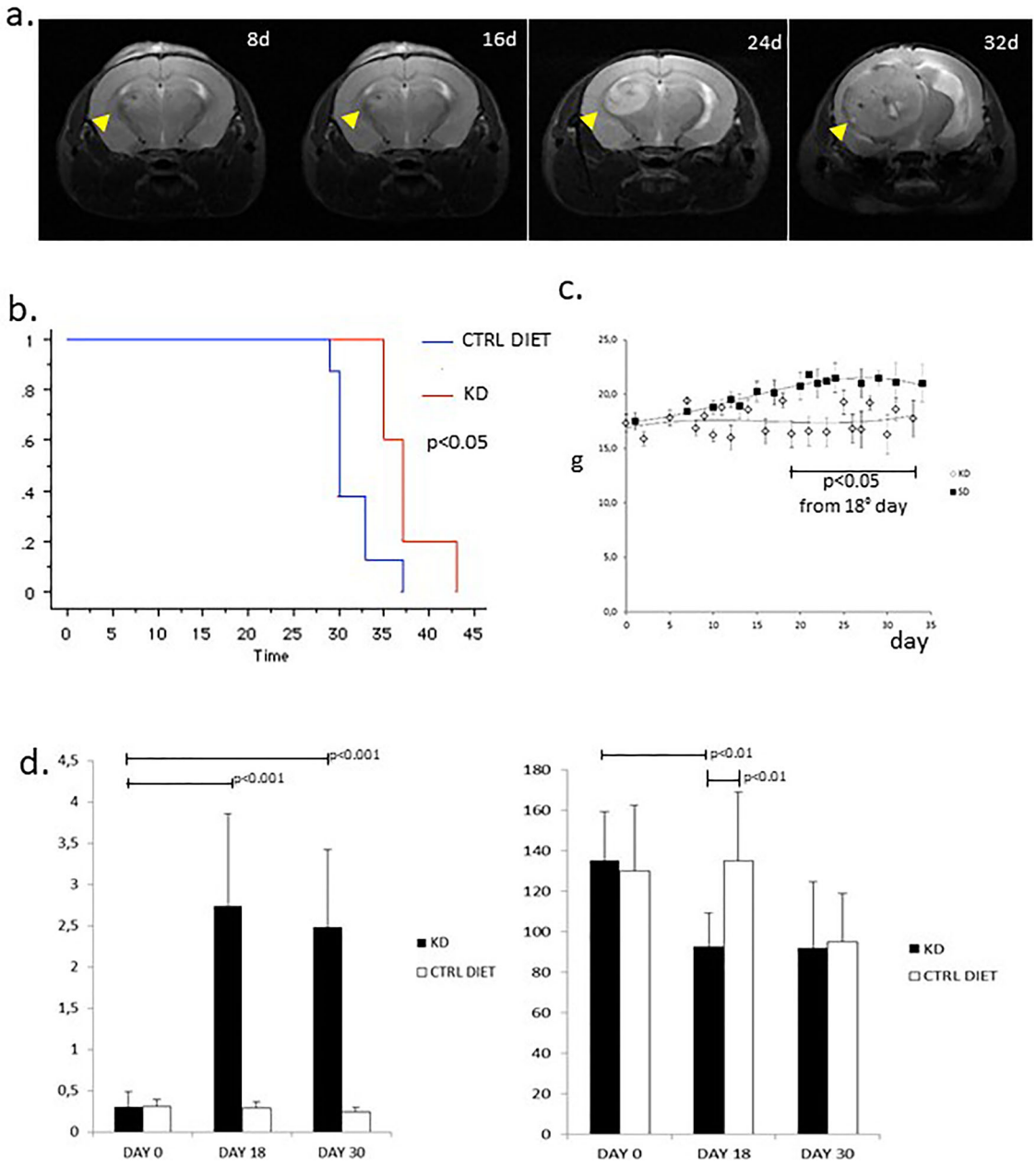

Figure 1. Mice intracerebrally injected with GL261 cells developed high grade gliomas in the designed cerebral region (a, yellow arrows). The panel shows the increasing size of the high grade glioma in the same animal at different time points (8-16-24 and 32 day); b) Kaplan Mayer survival curve of high grade glioma bearing mice fed on standard diet (CTRL diet) and ketogenic died (KD); c) Weight of healthy mice fed on standard diet and KD; d) ketogenic shift to ketone bodies metabolism: mice fed on KD (black bars) had increased ketone bodies in blood at different timepoints (left). They also showed a decrease of glucose blood levels even if they remained in the normal range value (right).

We also measured the *in vivo* switch to ketogenic metabolism by measuring ketone bodies and glucose concentration in blood at different time points. Figure 1d shows the relevance of the ketogenic shift *in vivo*, which peaked at day 18 and remained elevated until day 30 (black bars, left panel). This shift paralleled a significant reduction of blood glucose levels, which remained within the normal range (black bars, right panel). No statistically significant changes in glucose or ketone bodies were observed in mice fed with standard diet (Figure 1d, empty bars).

Spectroscopical Effects of KD on Brain Metabolism

MRI spectra of tumor bearing mice undergoing the KD were analyzed for absolute quantification of metabolites in tumor and contralateral normal brain tissue (Figure 2a).

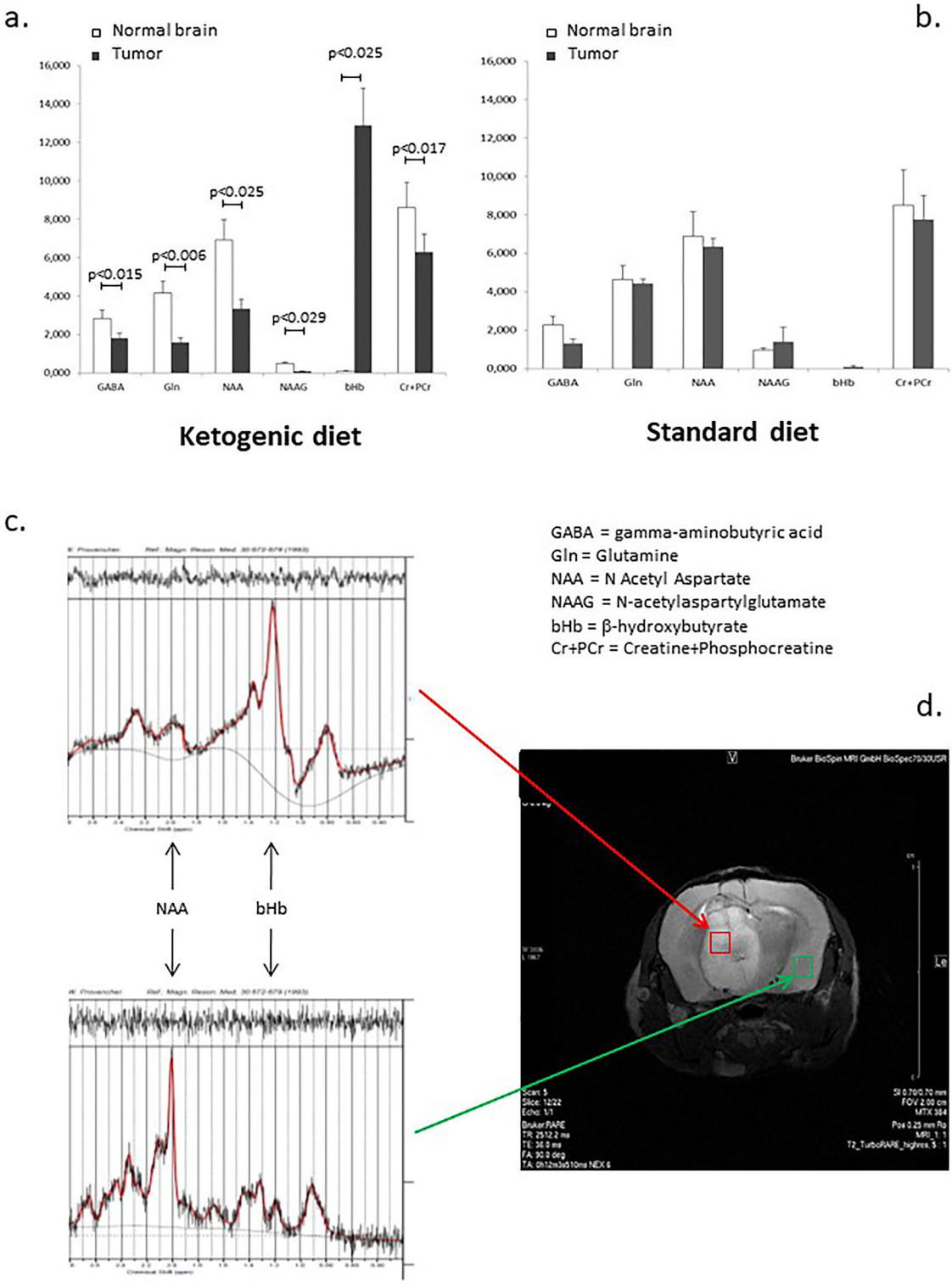

Figure 2. Differences in metabolic demand between tumor tissue and controlateral normal brain. At 23rd day after tumor cells injection, in mice fed with KD decreased concentrations of GABA, NAA and NAG were found in tumor tissue compared to normal brain. Gln and Cr + PCr were also decreased while a huge increase in bHb was detected in tumor tissue compared to normal brain (panel a). Quantification of the same parameters in mice fed with standard diet did not show any statistically significant difference between tumor tissue and controlateral brain (panel b). Data are referred to at least three different animals and are displayed as absolute concentration ± standard deviation. Examples of MRI spectra obtained at 23rd day from the beginning of the ketogenic diet intake are shown in tumor tissue and contralateral normal brain (panel c). The differences in peak height of bHB (increased in tumor tissue) and NAA (increased in normal brain tumor) is clearly evident. MRI image detailing the areas selected for spectroscopic analysis is also shown (panel d).

A significant decrease in concentrations of GABA, NAA and NAG were found in tumor tissue (data not shown) after 9 days from the beginning of the KD (2 weeks from tumor cell injection). At day 23, GABA, NAA and NAAG concentrations were still decreased in tumor tissue (Figure 2a). At this time point, Gln and Cr + PCr were also decreased while a huge increase in bHB was detected in tumor tissue compared to normal brain tissue (Figure 2a). Mice fed with standard diet did not display significant differences in terms of metabolite concentrations between tumor tissue and contralateral normal brain (Figure 2b). However, differently from mice fed with KD, no bHB peak was detected either in the tumor nor in contralateral normal brain.

Examples of MRI spectra obtained on the 23rd day from the start of the experiment are shown in Figure 2c (tumor tissue) and Figure 2d (contralateral normal brain). The differences in peak height of bHB (increased in tumor tissue, Figure 2b) and NAA (increased in normal brain tumor) are clearly shown.

Discussion

In our experimental model, non-restricted KD was able to induce a slight but statistically significant increase in survival rate in mice being actively treated as compared to the control group.

Our results closely resemble those reported by Stafford and coworkers that used unrestricted KD in the same animal model and were able to detect a short but statistically significant increase in survival [21].

Concerning investigation of metabolites by spectroscopy, the accumulation of bHB observed in the tumor tissue of KD-treated mice suggests that, in our experimental conditions, the elevated metabolic needs of growing tumor cells might not be supported by nutrients availability with consequent production of ketone bodies. This bHB accumulation, paralleled by the observation that GL261 cells *in vitro* do not display significant proliferation when exposed to bHB (unshown), suggests limited ability of tumor cells in this context to use it in mitochondrial metabolism. Similar *in vitro* data have been obtained by Maurer using other human glioma cell lines and by de Feyter using R2G and 9 L rat glioma cell lines [17, 24]. Moreover, the significantly prolonged survival rate detected in tumor-bearing mice fed with KD in our experimental conditions, further supports the hypothesis that glioma cells cannot use ketone bodies for their mitochondrial metabolism. Therefore, the huge amount of bHB detected locally might also reflect an effort by the tumor, not necessarily successful, to support its ongoing growth [17].

Although we cannot exclude that in our hands GL261 cells may have at least partly acquired the ability *in vivo* to metabolize bHB, it is tempting to speculate that the combination of hampered ketone body metabolism and relative hypo-glycemia, may have contributed to the increased survival in our experimental conditions. Previously published clinical studies are in agreement with this hypothesis showing that GBM patients with lower glycemia live longer in comparison with patients with higher glucose levels [28,29].

However, *in vivo* experiments conducted by De Feyter et al., using two rat glioma models, elegantly show increased transport and oxidation of bHB using a caloric-restricted KD [24]. Since we did not perform an *in vivo* metabolic evaluation as in their paper, we cannot exclude that the elevated bHB levels in the tumor tissue may concur to an effective tumor metabolism *in vivo*.

Taking into account the differences in outcome of KD-based treatments in different animal models, it is of paramount importance to assess *in vivo* putative shifts in bHB metabolism in patients undergoing KD. In a recent clinical study in which GBM patients were investigated serially by MRS, patients undergoing KD (in addition to bevacizumab) were compared with patients not partaking in the KD [30]. Acetone and acetoacetate were detectable in tumor tissue in 4 MR spectra, 4 and 25 mo respectively post initiation of the KD, while these peaks were not detected in patients not receiving the KD (bHB peaks were not reported in the paper) [30]. In agreement with these results, we did not detect ketone body peaks in the context of the tumor in mice fed with standard diet.

Moreover, in our experiments, no caloric restriction was applied, thus possibly minimizing the difference in outcome between the two groups. As a matter of fact, in vivo experiences in animal glioma models have shown a more profound anti-tumor activity in animals treated with caloric restriction in association with KD as compared with animals fed with unrestricted KD alone [12, 31].

Concomitantly with the ketogenic shift, we also observed a statistically significant reduction in blood glucose levels in mice fed with KD. Although the biological relevance of this observation needs to be further investigated, this suggests that glucose reduction, even if within the physiological range, may have an impact on glioblastoma growth and GBM-related

survival [32]. Reduction in glucose blood levels is an expected consequence of KD even in the absence of caloric restriction, and it has been shown to occur even in glioblastoma patients concurrently receiving steroids [24]. Moreover, clinical studies have reported reduced survival in steroid-treated glioblastoma patients and this reduction in survival is at least partly accounted for by steroid-induced hyperglycemia [29, 33–35].

The data obtained in our work provides additional evidence to support a possible role for KD as an adjunct treatment in the management of high grade glioma.

Translation of *in vitro* and *in vivo* studies for the treatment of human cancer is still pending; a recent review [36] supports a positive effect of KD in malignant glioma, however data from larger, carefully planned phase II trials are still needed, including QoL assessment and evaluation of long-term diet compliance, since palatability is still likely to be a relevant and unresolved issue for the long-term care in these patients.

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Disclosure Statement

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