Article

Profiling of Carnitine Shuttle System Intermediates in Gliomas Using Solid-Phase Microextraction (SPME)

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Abstract: Alterations in the carnitine shuttle system may be an indication of the presence of cancer. As such, in-depth analyses of this pathway in different malignant tumors could be important for the detec-tion and treatment of this disease. The current study aims to assess the profiles of carnitine and acylcarnitines in gliomas with respect to their grade, the presence of isocitrate dehydrogenase (IDH) mutations, and 1p/19q co-deletion. Brain tumors obtained from 19 patients were sampled on-site using solid-phase microextraction (SPME) immediately following excision. Analytes were desorbed and then analyzed via liquid chromatography-high resolution mass spectrometry. The results showed that SPME enabled the extraction of carnitine and 22 acylcarnitines. An analysis of the correlation factor revealed presence of two separate clusters: short-chain and long chain car-nitine esters. Slightly higher carnitine and acylcarnitine concentrations were observed in the higher malignancy tumor samples (high vs low grade), and in those samples with worse projected clinical outcomes (without vs with IDH mutation; without vs with 1p/19q co-deletion). Thus, the proposed chemical biopsy approach offers a simple solution for on-site sampling that enables sample preservation, thus supporting comprehensive multi-method analyses.

Keywords: glioma, cancer, carnitine, acylcarnitine, solid-phase microextraction SPME, liquid chromatog-raphy-mass spectrometry LC-MS

1. Introduction

Gliomas are among the most dangerous and insidious brain tumors due to their high heterogeneity and the late manifestation of a wide range of non-specific symptoms, such as seizures, headaches, nausea, dizziness, fatigue, vision problems, and numbness [1–4]. Delayed diagnosis favors tumor progression and leads to worse prognoses, and, consequently, a rapid decrease in the patient's quality of life. As a consequence, the introduction of accurate medical interventions, which often combine neurosurgery and chemo- or radiotherapy, is necessary. The selection of the best treatment is mainly based on the combined results of a histopathological examination and genetic and immunochemical testing. Genetic testing—for example, those that analyze the status of O6-methylguanine-DNA methyltransferase (MGMT) methylation, the presence of IDH mutation, or 1p/19q co-deletion—serves as a complement to the diagnosis process and enables accurate clinical prognoses [2]. Nonetheless, the survival rate of glioma patients is still low due to a lack

of effective treatment methods [1–4]. Thus, it is critical to further expand our basic knowledge about the metabolism of these tumors, as such information is indispensable in improving clinical prognoses and the effectiveness of treatments.

Cancer cells are characterized by increased metabolism, which generates ATP, NADPH, and other intermediates for tumor growth, as well as high adaptability to the dynamically changing microenvironment [3,5]. For a long time, the so-called Warburg effect – wherein increased aerobic glycolysis results in enhanced lactate production, rather than pyruvate production – has been cited as the main source of energy production in gliomas and other neoplasms [5]. However, recent findings suggest that altered fatty acid oxidation (FAO) is also an important marker of glioma initiation and development [3,5–7]. This metabolic pathway is mainly regulated by the carnitine shuttle system, which consists of enzymes and protein transporters that are responsible for transporting fatty acids through the mitochondria's membrane [5]. Although the expression and enzymatic activity of this pathway's protein components (e.g., carnitineacylcarnitine translocase (CACT), carnitine palmitoyltransferase I (CPT-1) or carnitine palmitoyltransferase II (CPT-2), and long-chain acylocarnitines dehydrogenase (LCAD)) have been extensively studied [3,5], the role and fate of carnitine and the esters (also known as acylcarnitines) produced from transporting fatty acids though the mitochondrial membrane remain unclear.

There are many methods that can be used to analyze changes in chemical composition of tissues, such as homogenization followed by liquid-liquid extraction, microdialysis, and solid-phase microextraction (SPME) [8–11]. SPME, which is based on the interaction between an active sorbent and targeted substances dispersed in a given matrix, offers a number of significant advantages. One of the most notable of these advantages is SPME's ultra-simple sampling procedure, which enables samples to be acquired directly from tissues without any major structural disruption. In addition, the SPME protocol can be implemented on-site (i.e., surgery room) by medical personnel who have no analytical background. The most common SPME device is a thin nickel-titanium fiber coated with an active sorbent to a final diameter of ca. 250 μm. Under the SPME protocol, the probe is first introduced into the tissue for a time period that has been predetermined to enable optimal metabolite binding, followed by desorption of the analytes from the device into an organic solvent. It should be emphasized that the extracted analytes do not require any additional treatment prior to instrumental analysis on an LC-MS platform. This methodology has been characterized in greater detail in the literature [8,12].

Our previous work on the untargeted metabolomic characterization of brain tumors found characteristic differences in metabolome composition with regards to histological type and genetic aberrations [12–14]. However, (semi)quantitative targeted analysis would provide more accurate information about the biochemical changes in a cancerous tissue. Therefore, this study aims to develop a more in-depth understanding of the carnitine shuttle system's intermediates using data acquired from untargeted lipidomic analyses of brain tumors via SPME-LC-MS, with particular consideration given to tumor grade, the presence of IDH mutation, and 1p/19q codeletion.

2. Results and discussion

The use of multiplatform studies combining various analytical methods – for example, extending routine tests to –omic studies – has become more common in basic cancer research [15], as such approaches are able to provide detailed analysis of the metabolic pathways and identify tumor vulnerability [16]. However, combining various techniques to analyze a single sample also faces limitations due to small amounts of sample, and analyte instability. Given these limitations, it is critical to continue to explore other approaches to sampling. The methodology of profiling carnitine and its esters proposed herein is based on non-sample-consuming sampling, which is an approach that could enable additional testing (e.g., chemical biopsy followed by genetic testing or histopathology examination of the same specimen).

Carnitine is integral to the proper functioning of the enzymes (CPT-1, CPT-2, CACT) involved in transporting long chain fatty acids across the inner mitochondrial membrane. Thus, this metabolite is considered a crucial regulator of the carnitine shuttle system [5,17]. Our findings showed that the concentration of FC was significantly higher in HGG compared to LGG, and in IDHw compared to IDHm, with fold change of 4.21 and 3.91, respectively (Table 1). Although no significant difference was observed in the tumor carnitine levels of patients with and without the presence of 1p/19q co-deletion, the area under the peak was higher for patients with tumors not featuring co-deletion. Prior studies have examined levels of this metabolite in a variety of malignant neoplasms, including glioma, hepatocellular carcinoma, breast cancer, and prostate cancer, with findings showing higher concentrations in malignant tissues compared to histologically healthy samples [5,10,11,18,19]. In our study, we did not compare healthy and cancerous samples; rather, we compared cancerous samples with varying grades, with results indicating higher carnitine content in higher grade tumors. Furthermore, our results agreed with a prior comprehensive metabolomic analysis of mutant and wild-type samples, which revealed higher carnitine concentrations in glioblastoma cells without the IDH mutation [17]. The higher levels of carnitine detected in HGG and IDHw could be related to the increased metabolism of these tumors compared to LGG and IDHm, which is consistent with Melone et al.'s [5] model of cancer metabolism. The carnitine shuttle system plays an important role in cancer plasticity, and it enables the metabolic demands of proliferating cancer cells to be fulfilled, even in adverse conditions.

The use of SPME-LC-HRMS for acylcarnitine analysis enabled the identification of 22 simple-chain saturated and unsaturated acylcarnitines (AC) (Table 1): five short-chain acylcarnitines (AC C2 – AC C5, SCAC), seven medium chain acylcarnitines (AC C6 – AC C12, MCAC), and ten long-chain acylcarnitines (AC C14 – AC C20, LCAC). The acylcarnitines identified in our study have also been observed by other researchers in their work on different types of malignant neoplasms (e.g., prostate, kidney, or liver); however, these prior works used sample-preparation protocols that are more complex than SPME [10,19,20]. For instance, prior works have mainly used tissue homogenization, which is a sample-consuming approach, followed by liquid-liquid extraction prior to instrumental analysis. Thus, this method precludes the re-use of the sample with other diagnostic approaches. Moreover, the SPME approach used in the present work combines sampling and extraction into a single step, which greatly simplifies the sample-preparation procedure. The utility of this non-sample-consuming technique for tissue analysis has been widely reported elsewhere [8,21–23].

Table 1. Carnitine and acylcarnitines identified in gliomas sampled via solid-phase microextraction (SPME). Table represents identification details and ratios of peak areas for detected analytes FC – free carnitine; AC – acylcarnitine; m/z – mass-to-charge ratio; RT – retention time; LGG – low-grade glioma; HGG – high-grade glioma; IDHm – IDH mutation, IDHw – IDH wildtype; del – presence of 1p/19q co-deletion; ndel – absence of 1p/19q co-deletion

AC	Chemical formula [M+H+]	M/Z [M+H$^+$]	RT [min]	HGG: LGG	IDHw: IDHm	n-del: del
FC	$C_7H_{16}O_3N_1$	162.1126	15.60	4,21[a]	3,91[b]	3,37
AC C2:0	$C_9H_{18}O_4N_1$	204.1231	12.46	2.97	2.72	2.52
AC C3:0	$C_{10}H_{20}O_4N_1$	218.1387	11.00	11.22[a]	3.12	5.64[c]
AC C4:0	$C_{11}H_{22}O_4N_1$	232.1543	9.72	3.60	2.42	3.51
AC C5:1	$C_{12}H_{22}O_4N_1$	244.1543	9.28	3.42	1.96	3.40
AC C5:0	$C_{12}H_{24}O_4N_1$	246.1700	8.96	6.37	1.68	6.58
AC C6:0	$C_{13}H_{26}O_4N_1$	260.1856	8.37	3.78	2.18	3.26
AC C8:0	$C_{15}H_{30}O_4N_1$	288.2169	7.79	6.13	1.56	4.55

AC C9:0	$C_{16}H_{32}O_4N_1$	302.2325	7.62	134.21[a]	4.06	11.08
AC C10:1	$C_{17}H_{32}O_4N_1$	314.2326	7.52	11.00	2.51	4.56
AC C10:0	$C_{17}H_{34}O_4N_1$	316.2484	7.48	8.41	1.78	5.86
AC C12:1	$C_{19}H_{36}O_4N_1$	342.2640	7.27	6.63	2.11	3.91
AC C12:0	$C_{19}H_{38}O_4N_1$	344.2796	7.23	7.78	2.18	4.79
AC C14:2	$C_{21}H_{38}O_4N_1$	368.2797	7.12	10.31	1.35	4.42
AC C14:1	$C_{21}H_{40}O_4N_1$	370.2953	7.07	6.80	1.29	4.07
AC C14:0	$C_{21}H_{42}O_4N_1$	372.3108	7.10	4.07	1.59	2.62
AC C16:1	$C_{23}H_{44}O_4N_1$	398.3266	6.96	3.81	0.74	1.91
AC C16:0	$C_{23}H_{46}O_4N_1$	400.3423	6.96	4.04	1.18	2.51
AC C18:2	$C_{25}H_{46}O_4N_1$	424.3422	6.89	6.00	0.79	2.27
AC C18:1	$C_{25}H_{48}O_4N_1$	426.3579	6.84	5.01	0.87	2.80
AC C20:4	$C_{27}H_{46}O_4N_1$	448.3424	6.80	12.46	2.60	5.15
AC C20:3	$C_{27}H_{48}O_4N_1$	450.3578	6.78	69.87	2.70	7.72
AC C20:1	$C_{27}H_{50}O_4N_1$	454.3891	6.71	10.63	8.29	22.15

[a] the average peak area for HGG is statistically significantly different from LGG, $p<0.05$
[b] the average peak area for IDHw is statistically significantly different from IDHm, $p<0.05$
[c] the average peak area for n-del is statistically significantly different from del, $p<0.05$

To explore the relationship between carnitine and its particular esters in all of the obtained glioma samples, a correlation clustering analysis was performed, with the results revealing a high correlation factor for specific patterns of acylcarnitines (Fig. 1). The analytes classified as SCAC represented one correlation cluster, with a correlation coefficient above or equal to 0.63, while MCAC and LCAC were correlated with each other, with a minimum factor of 0.51. Moreover, no clear correlation was observed between the metabolites with short- and long-acyl-chain analytes. Lu et al. [10] obtained similar results in their attempt to profile acylcarnitines in liver cancer, namely, that LCAC and SCAC formed separate correlation clusters. In addition, it was difficult to clearly define the correlation clusters for MCAC due to its varying dependency on the carnitine shuttle system. This phenomenon could be the result of the dependence of long-acyl-chain acylcarnitines on the enzymes in the carnitine shuttle system. Due to their small size, acylcarnitines with short acyl-chains are able to pass through the mitochondrial membrane without the support of CPT-1, CPT-2, and LCAD, whereas the passage of LCAC is strictly controlled by the carnitine shuttle system [3]. The MCAC transport system is supposed to fall in between these two regulations.

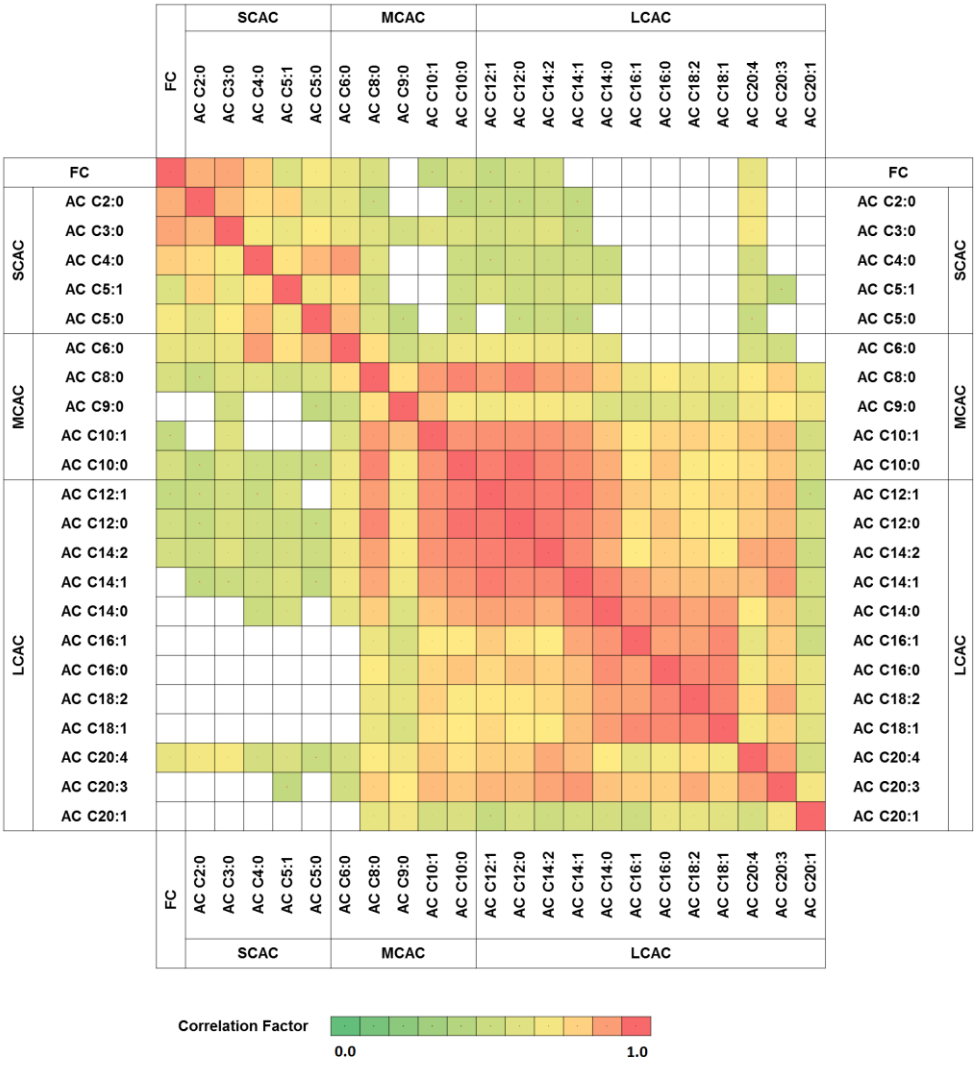

Figure 1. Carnitine and acylcarnitines correlation network. Only statistically significant correlation factors were presented ($p<0.05$)
FC – free carnitine; AC – acylcarnitine; SCAC – short-chain-length acylcarnitines; MCAC – medium-chain-length acylcarnitines; LCAC – long-chain-length acylcarnitine

A comparative analysis of the acylcarnitine profiles in the different subgroups of the glioma samples was also conducted. To this end, the tumors were divided into groups based on their grade, IDH mutation status, and the presence of 1p/19q co-deletion. The results showed that the average peak areas for SCAC, MCAC and LCAC were larger for HGG than for LGG, although statistical significance was observed only for SCAC (Fig. 2.A). A detailed analysis of the identified acylocarnitines revealed that the level of a particular species in HGG was at least three times bigger than in LGG (Table 1). Once again, however, a statistically significant difference between HGG and LGG was observed for AC C3:0 and AC C9:0, with fold changes of 11.22 and 134.21, respectively (Table 1). The higher acylcarnitine concentration in the high-grade gliomas could be explained by activated proliferation and the higher rates of metabolism in malignant lesions. The findings of Kant et al.'s [3] study of FAO in gliomas showed that glioblastomas contained higher levels of acylcarnitines compared to low-grade astrocytomas, which could be due to enhanced activity among carnitine shuttle enzymes [3].

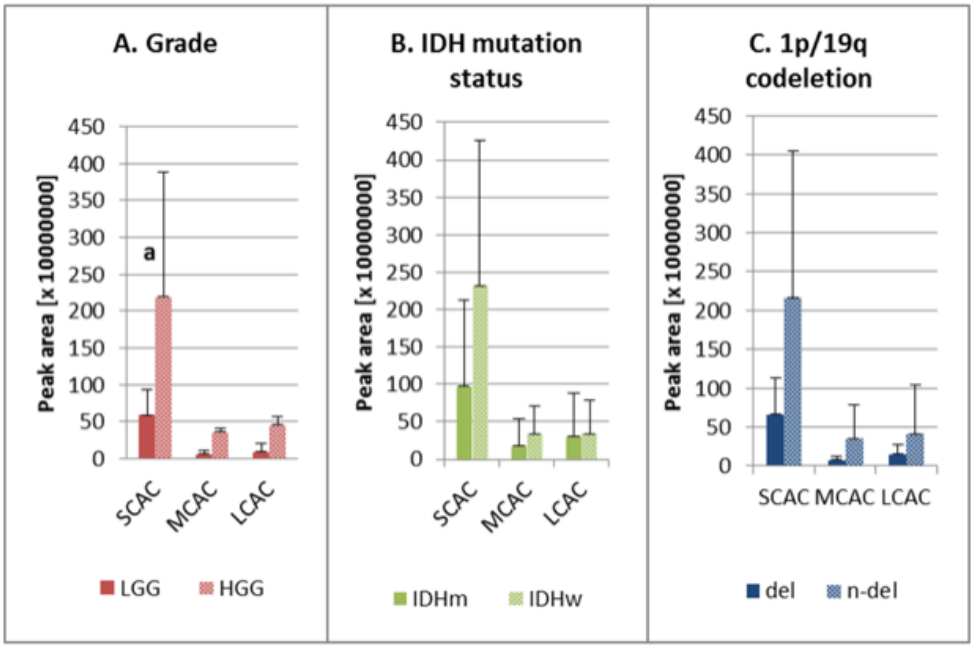

Figure 2. Ratios of SCAC, MCAC, and LCAC in the studied groups: A.) grade – low- (LGG) and high-grade glioma (HGG); B.) IDH mutation status – IDH mutant (IDHm) and IDH wildtype (IDHw); C.) 1p/19q co-deletion – presence of deletion (del) and absence of deletion (n-del)
SCAC – short-chain-length acylcarnitines; MCAC – medium-chain-length acylcarnitines; LCAC – long-chain-length acylcarnitine; AC – acylcarnitine; LGG – low-grade glioma; HGG – high-grade glioma; IDHm – IDH mutation, IDHw – IDH wildtype; del – presence of 1p/19q co-deletion; n-del – absence of 1p/19q co-deletion
[a] *the average peak area for HGG is statistically significantly different from LGG, p<0.05*

One of the main prognostic factors with respect to gliomas is the presence of a mutation in the gene encoding isocitrate dehydrohensase (IDH) [2], which is responsible for catalyzing the oxidative decarboxylation of isocitrate to 2-oxoglutarate in the tricarboxylic acid (TCA) cycle. During this process, nicotinamide adenine dinucleotide phosphate (NADP+) is reduced to nicotinamide adenine dinucleotide phosphate (NADPH) [24,25], which serves as a redox power for overcoming oxidative stress generated during cancerogenesis, as well as a co-enzyme in anabolic processes resulting in cellular proliferation. FAO by production of acetyl CoA, which is the TCA substrate, can be influenced by alterations related to IDH mutation [25]. Our findings revealed higher levels of SCAC, MCAC, and LCAC in the IDH wildtype samples (versus the mutated samples), although the difference was not statistically significant (Fig. 2.B). Furthermore, assessments of the peak areas for particular acylcarnitines also did not show any significant differences between IDHw and IDHm, which may be the result of the small cohort used in this study. Nonetheless, the values were higher in the samples obtained from wildtype samples in the majority of cases (Table 1). It is likely that IDH mutation leads to changes in the activity of the carnitine shuttle system, which in turn result in the observed metabolic changes. This assumption could help to improve the prognosis of IDHm patients, as the down regulation of fatty acid transport reduces the proliferation rate of cancer cells and, ultimately, tumor malignancy [2,26]. A similar observation has been reported by Miyata et al. [17], who also identified lower concentrations of carnitine and acetylcarnitine in IDH-mutant gliomas compared to the wildtype variants. On the other hand, Kant et al. [3] did not observe any major differences in the amount of detected carnitine and its esters.

Testing aimed at detecting the co-deletion of chromosomes 1p and 19q and IDH mutation is one of the main genetic approaches to glioma diagnosis, as it enables the differentiation of oligodendriogliomas from astrocytomas [2]. In our study, the peak areas for all acylcarnitines were not significantly larger in the samples without the chromosomal

aberration (Fig. 2.C). The analysis of particular compounds revealed a significantly greater peak area only for AC C3:0, and a ratio between the studied groups (n-del:del) of 5.64 (Table 1). It was not possible to explain the direct biochemical correlation between lipid metabolism and the presence of 1p/19q co-deletion based on the available literature. However, it is worth mentioning that the patients without this aberration had poorer responses to radiotherapy and worse survival rates compared to patients with the 1p/19q co-deletion [27]. Therefore, slightly higher levels of carnitine intermediates in wild type might help to explain different responses to radiotherapy among patients with diverse status of this aberration in the future.

The content of carnitine and its esters in glioblastoma samples cannot provide comprehensive information about changes in FAO on their own. Therefore, we calculated additional indicators that enable carnitine shuttle system action to be estimated. For example, calculating the oxidation rate of even- and odd-numbered fatty acids makes it possible to assess lipid transformation and energy production [11, 20, 28]. In addition, the activity of crucial carnitine shuttle system enzymes such as CPT-2 and LCAD were also evaluated on the basis of appropriate acylcarnitines ratios [11,20].

Our study revealed that, compared to HGGs, LGGs were characterized by significantly higher AC C2:0 to FC ratios. In contrast, the ratio of AC C3:0 to FC was 2.4 times higher in more malignant tumors, though the p-value was above 0.05 (Table 2). Yu et al. [11] used the same formulas that we used in this study to estimate the FAO rate in glioblastomas, and found that the oxidation of even-numbered fatty acids was not dependent on tumor grade. However, the ratio of AC C3:0 to FC was significantly higher for the more malignant lesions, which is in agreement with our results. Conversely, research conducted on hepatocellular carcinoma (HCC) serum samples found no statistically significant differences in the ratio of AC 2:0 to FC among cancerous and non-cancerous patients [28].

Estimations of the activity of enzymes related to carnitine transformation (i.e., CPT-2 and LCAD) revealed no significant differences between HGG and LGG patients (Table 2). Similar results were reported by Yu et al. [11], who estimated no significant differences in activity among CPT-1 and CPT-2 in different grades of glioblastoma samples. It should be noted that, despite the lack of statistical differences, we found that CPT-2 activity was almost three times higher in HGGs than in LGGs. This finding is consistent with Melone et al.'s [5] results, which demonstrated that this enzyme has higher activity levels in cancerous samples than in non-cancerous samples. On the other hand, LCAD activity was 2.2 times lower in HGG than in LGG, though the difference was not statistically significant. According to previous studies [29], the depletion of LCAD is likely to promote the proliferation of cancer cells, thereby increasing the mortality rates of patients.

Table 2. Ratios of selected acylcarnitines and carnitne used to estimate the activity of enzymes related to the carnitine shuttle system
AC – acylcarnitine; LGG – low-grade glioma; HGG – high-grade glioma; IDHm – IDH mutation, IDHw – IDH wildtype; del – presence of 1p/19q co-deletion; n-del – absence of 1p/19q co-deletion; SD – standard deviation; FC – free carnitine

Estimated process	**LGG**		**HGG**		**IDHm**		**IDHw**		**del**		**n-del**	
	Average	**SD**	**average**	**SD**	**average**	**SD**	**average**	**SD**	**average**	**SD**	**average**	**SD**
Carnitine palmitoyltransferase 2 (CPT-2) activity	**0.19**	0.30	**0.53**	1.04	**0.62**	1.15	**0.17**	0.16	**0.72**	1.36	**0.22**	0.27
Long-chain Acyl-CoA dehydrogenase (LCAD) activity	**4.50**	5.27	**2.01**	2.47	**4.45**	4.70	**1.24**	1.27	**2.77**	3.06	**3.02**	4.31

The rate of oxidation of even-carbon fatty acids	**1.53**[a]	0.50	**0.97**[a]	0.34	**1.41**[b]	0.50	**0.92**[b]	0.34	**1.31**	0.43	**1.10**	0.52
The rate of oxidation of odd-carbon fatty acids	**0.05**	0.04	**0.12**	0.09	**0.10**	0.11	**0.09**	0.03	**0.06**	0.06	**0.11**	0.08

[a] the average peak area for HGG is statistically significantly different from LGG. $p<0.05$
[b] the average peak area for IDHw is statistically significantly different from IDHm. $p<0.05$

The FAO rate and CPT-2 and LCAD activity were also assessed in relation to the presence or absence of the IDH mutation. The findings showed that the ratio of AC C2:0 to FC was significantly higher in the IDHm samples, while the parameter characterizing the metabolism of odd-numbered fatty acids was not distinct for the studied groups. Calculations for the other ratios used estimate CPT-2 and LCAD activity did not reveal significant differences between IDHm and IDHw. However, it should be noted that these parameters were slightly higher in the mutant samples (Table 2).

In case of the presence or absence of 1p/19q co-deletion, no significant changes in the calculated factors were observed (Table 2). Nonetheless, CPT-2 activity was estimated to be 3.3 times higher for brain tumors with 1p/19q co-deletion, while LCAD activity was estimated to be marginally lower in these samples.

On the whole, our results suggest that even-carbon fatty acids have a higher oxidation rate in patients with better clinical prognosis (LGG and IDHm) [2]. This factor was also slightly higher in the samples with 1p/19q co-deletion, but this increase was not significant. Thus, our earlier assumption regarding the low discriminating power of this aberration was confirmed. Additionally, no statistically significant differences were found in the oxidation rate of odd-carbon fatty acids, as well as CPT-1 and LCAD activity, between the compared groups. However, the peaks differed slightly between the studied groups, which could indicate that the grade or presence of a genetic aberration could be related to alterations in carnitine shuttle system activity. Nevertheless, due to high heterogeneity among brain tumors (i.e., high standard deviation) (Table 2) and the relatively small group of participants in this study, it is not possible to draw solid conclusions and only patterns of changes could be discussed. Therefore, this experiment should be repeated using a bigger population, and complemented with immunochemical analysis of the activity of enzymes in the carnitine shuttle system. However, the current study clearly showed that application of the untargeted approach based on SPME-LC-HRMS enabled comprehensive screening analysis of all carnitine esters indicating validity of their further in-depth targeted quantitative analysis.

The results of this study indicate that alterations in the carnitine shuttle system might be an important factor in estimating glioma malignancy and assessing clinical prognosis. The use of SPME combined with LC-HRMS enabled carnitine and 22 of its esters to be profiled in glioma samples. Our findings also revealed that SCAC and LCAC formed clearly separated correlation clusters, which could indicate their different levels of dependence on the carnitine shuttle system. Moreover, we were able to profile carnitine and acylocarnitines in glioma samples, and estimate the activity of LCAD and CPT-2, as well as the oxidation rate of fatty acids in studied tissues. We observed that the content of FC and acylcarnitines was usually higher in higher-malignancy tumors (HGG vs LGG) or in patients with worse clinical outcomes (IDHw vs IDHm and with 1p/19q co-deletion vs without 1p/19q co-deletion). The oxidation rate and carnitine shuttle enzyme activity were also estimated, with findings showing slight alterations in the studied groups. This result suggests changes in the carnitine shuttle system during cancerogenesis, and in the presence of genetic aberrations. However, it was not possible to form any firm conclusions about our results due to the high heterogeneity among the studied samples, and the lack

of results obtained using reference methods (e.g., using immunochemistry to assess enzyme activity). In the future, it may be possible to obtain more detailed information about the biology of brain tumors by combining SPME with histological, immunochemical, or genetic platforms. Regardless, this research demonstrates that SPME can be very simple solution for more complex and comprehensive studies due to its no-sample consuming nature.

3. Materials and Methods

Biological Material

Brain tumors were obtained via neurosurgical procedures conducted at the 10th Military Research Hospital and Polyclinic in Bydgoszcz, Poland. Overall, 19 samples were analyzed. The characteristics of these samples were as follows: 7 were low-grade gliomas (LGG) and 12 were high-grade gliomas (HGG); 10 were IDH mutant (IDHm) tumors, and 9 were IDH wildtype (IDHw) tumors; and 7 featured 1p/19q co-deletion (del), while 12 did not (n-del). A detailed summary of the patients' characteristics is presented in Table S1 in the Supplementary Materials. The study was approved by the Bioethical Committee in Bydgoszcz, Poland (KB 628/2015).

Chemical Biopsy (Solid-Phase Microextraction) Protocol

Sampling was conducted using 7 mm C18 fibers kindly provided by Supelco, Merck, immediately following the removal of the brain tumor. To this end, the protocol developed by Bogusiewicz et al. [12] was employed, with minor modifications. The fibers were preconditioned overnight in a methanol:water (1:1 v/v) solution to activate the sorbent, and were rinsed with water directly before sampling in order to remove any organic solvent residue. The fibers were then inserted into the tumor tissue for 30 minutes, and then rinsed again in water to remove any residues from cell debris or blood. Next, the fibers were stored in a freezer at -30° C until desorption (1hour), which was conducted in silanized glass vials with 150µl of an isopropanol:methanol (1:1 v/v) solution and agitation at 1200 rpm. Pooled quality control (QC) and extraction blanks (negative control) were also prepared.

Instrumental Analysis

The liquid chromatrography-high resolution mass spectrometry (LC-HRMS) platform consisted of a Dionex UltiMate 3000 RS autosampler, a Dionex Ultimate 3000 RS pump (Thermo Fisher Scientific, Dionex, Germany), and a QExactive Focus high-resolution mass spectrometer (Thermo Fisher Scientific, Germany).

LC analysis was conducted using the following parameters: phase A – 5 mM ammonium acetate in water; phase B – acetonitrile; gradient – 0-2 min 96% B, 15.0 min 80% B, 15.1-21.0 min 96% B; SeQuantZIC-cHILIC – 3 µm 100 x 2.1 mm column; flow – 0.4 mL/min; oven temperature – 40 °C; and injection volume – 10 µL.

The present study used positive ion mode with the following parameters: a scan range of 100-1000 m/z; acquisition via AGC (1,000,000 ions); a spray voltage of 1.5 kV; an S-lens RF level of 55%; an S-lens voltage of 25 V; a skimmer voltage of 15 V; a capillary temperature of 325 °C; sheath gas at 60 a.u.; aux gas at 30 a.u.; spare gas at 2 a.u.; and a probe heater temperature of 320 °C. Only acylcarnitines in the extraction QC samples with a coefficient of variation (CV) of less than 10% were accepted for analysis. These

acylcarnitines were identified by matching their fragmentation patterns with spectra libraries at a mass accuracy of <3ppm (the presence of characteristic fragment: 85.0290 peak in MS/MS spectra). Full MS/dd-MS2 discovery mode was used for this purpose. Matching was conducted using the following fragmentation parameters: mass resolution—35000 FWHM; AGC target—2E4; minimum AGC—8E3; intensity threshold—auto; maximum IT—auto; isolation window—3.0 m/z; stepped collision energy—20V, 30V, 40V; loop count—2; and dynamic exclusion—auto.

Data Processing and Statistical Analysis

Acylcarnitine identification was performed using LipidSearch 4.1.30 (Thermo Fisher Scientific, San Jose, California, USA) software, which is capable of identifying simple-chain carnitine esters with eight or more carbons in their structure (AC C8:0) (Table S2 in the Supplementary Materials). As such, carnitine and acylcarnitines with shorter chains were searched manually using mzCloud and the Human Metabolome Database (HMDB).

Statistical analysis was conducted using Statistica 13.3 PL (StatSoft, Inc., Tulsa, Oklahoma, USA) software. The average peak area for all analytes was calculated, and statistical tests were applied. In particular, Levene's test was used to assess variation, and the Shapiro-Wilks test was applied to assess normality. A T-test was subsequently applied when variation was homogenous and the variables were normal, while the Mann-Whitney U Test was used in all other cases.

Ratios of selected acylcarnitines and carnitine were calculated in order to estimate the activity of enzymes related to the carnitine shuttle system [11]. The activity of CPT-2 was assessed based on the ratio of (AC C16:0 + AC C18:1) to AC C2:0, while the ratio of AC C16:0 to AC C8:0 was used to estimate the activity of long-chain Acyl-CoA dehydrogenase (LCAD). The oxidation rates of even- and odd-carbon fatty acids were analyzed based on the ratios of AC C2:0 to free carnitine (FC) and AC C3:0 to FC, respectively. It was not possible to estimate the activity of CPT-1 due to a lack of MS/MS confirmation of the presence of AC C18:0, which is used in the calculation formula: (AC C16:0 + AC C18:0) / FC.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: title, Table S1: title, Video S1: title.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Bioethics Committee of Collegium Medicum in Bydgoszcz at Nicolaus Copernicus University in Toruń (KB 628/2015; on Sept 29, 2015).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Not applicable.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Table S1 Detailed description of samples included in the study; Table S2 Acylcarnitines which could be identified using LipidSearch

References

1. Lin, H.; Patel, S.; Affeck, V.S.; Wilson, I.; Turnbull, D.M.; Joshi, A.R.; Maxwell, R.; Stoll, E.A. Fatty acid oxidation is required for the respiration and proliferation of malignant glioma cells. *Neuro. Oncol.* **2017**, *19*, 43–54, doi:10.1093/neuonc/now128.
2. Louis, D.N.; Perry, A.; Reifenberger, G.; von Deimling, A.; Figarella-Branger, D.; Cavenee, W.K.; Ohgaki, H.; Wiestler, O.D.; Kleihues, P.; Ellison, D.W. The 2016 World Health Organization Classification of Tumors of the Central Nervous System: a summary. *Acta Neuropathol.* **2016**, *131*, 803–820, doi: 10.1007/s00401-016-1545-1.
3. Kant, S.; Kesarwani, P.; Prabhu, A.; Graham, S.F.; Buelow, K.L.; Nakano, I.; Chinnaiyan, P. Enhanced fatty acid oxidation provides glioblastoma cells metabolic plasticity to accommodate to its dynamic nutrient microenvironment. *Cell Death Dis.* **2020**, *11*, doi:10.1038/s41419-020-2449-5.
4. Duman, C.; Yaqubi, K.; Hoffmann, A.; Acikgöz, A.A.; Korshunov, A.; Bendszus, M.; Herold-Mende, C.; Liu, H.K.; Alfonso, J. Acyl-CoA-Binding Protein Drives Glioblastoma Tumorigenesis by Sustaining Fatty Acid Oxidation. *Cell Metab.* **2019**, *30*, 274-289.e5, doi:10.1016/j.cmet.2019.04.004.
5. Melone, M.A.B.; Valentino, A.; Margarucci, S.; Galderisi, U.; Giordano, A.; Peluso, G. The carnitine system and cancer metabolic plasticity review-article. *Cell Death Dis.* **2018**, *9*, doi:10.1038/s41419-018-0313-7.
6. Juraszek, B.; Czarnecka-Herok, J.; Nałęcz, K.A. Glioma cells survival depends both on fatty acid oxidation and on functional carnitine transport by SLC22A5. *J. Neurochem.* **2021**, *156*, 642–657, doi:10.1111/jnc.15124.
7. Strickland, M.; Stoll, E.A. Metabolic reprogramming in glioma. *Front. Cell Dev. Biol.* **2017**, *5*, doi:10.3389/fcell.2017.00043.
8. Reyes-Garcés, N.; Gionfriddo, E.; Gómez-Ríos, G.A.; Alam, M.N.; Boyacl, E.; Bojko, B.; Singh, V.; Grandy, J.; Pawliszyn, J. Advances in Solid Phase Microextraction and Perspective on Future Directions. *Anal. Chem.* 2018, *90*, 302–360, doi:10.1021/acs.analchem.7b04502.
9. Ngernsutivorakul, T.; Steyer, D.J.; Valenta, A.C.; Kennedy, R.T. In Vivo Chemical Monitoring at High Spatiotemporal Resolution Using Microfabricated Sampling Probes and Droplet-Based Microfluidics Coupled to Mass Spectrometry. *Anal. Chem.* **2018**, *90*, 10943–10950, doi:10.1021/acs.analchem.8b02468.
10. Lu, X.; Zhang, X.; Zhang, Y.; Zhang, K.; Zhan, C.; Shi, X.; Li, Y.; Zhao, J.; Bai, Y.; Wang, Y.; et al. Metabolic profiling analysis upon acylcarnitines in tissues of hepatocellular carcinoma revealed the inhibited carnitine shuttle system caused by the downregulated carnitine palmitoyltransferase 2. *Mol. Carcinog.* **2019**, *58*, 749–759, doi:10.1002/mc.22967.
11. Yu, D.; Xuan, Q.; Zhang, C.; Hu, C.; Li, Y.; Zhao, X.; Liu, S.; Ren, F.; Zhang, Y.; Zhou, L.; et al. Metabolic alterations related to glioma grading based on metabolomics and lipidomics analyses. *Metabolites* **2020**, *10*, 1–11, doi:10.3390/metabo10120478.
12. Bogusiewicz, J.; Zofiagoryńska, P.; Gaca, M.; Chmara, K.; Goryński, K.; Jaroch, K.; Paczkowski, D.; Furtak, J.; Harat, M.; Bojko, B. On-site sampling and extraction of brain tumors for metabolomics and lipidomics analysis. *J. Vis. Exp.* **2020**, *2020*, 159, doi:10.3791/61260.
13. Bogusiewicz, J.; Goryńska, P.Z.; Jaroch, K.; Goryński, K.; Paczkowski, D.; Furtak, J.; Harat, M.; Bojko, B. P13.05 Chemical Biopsy as an Alternative Sampling Method in Neurooncology. *Neuro. Oncol.* **2019**, *21*, doi:10.1093/neuonc/noz126.226.
14. Goryńska, P.Z.; Chmara, K.; Goryński, K.; Paczkowski, D.; Harat, M.; Bojko, B. A new strategy for brain tumour metabolomic analysis. *Med. Res. J.* **2018**, *3*, 15–22, doi:10.5603/mrj.2018.0003.
15. Menyhárt, O.; Győrffy, B. Multi-omics approaches in cancer research with applications in tumor subtyping, prognosis, and diagnosis. *Comput. Struct. Biotechnol. J.* 2021, *19*, 949-960, doi: 10.1016/j.csbj.2021.01.009.

16. Wang, H.; Diaz, A.K.; Shaw, T.I.; Li, Y.; Niu, M.; Cho, J.H.; Paugh, B.S.; Zhang, Y.; Sifford, J.; Bai, B.; et al. Deep multiomics profiling of brain tumors identifies signaling networks downstream of cancer driver genes. *Nat. Commun.* **2019**, *10*, doi:10.1038/s41467-019-11661-4.
17. Miyata, S.; Tominaga, K.; Sakashita, E.; Urabe, M.; Onuki, Y.; Gomi, A.; Yamaguchi, T.; Mieno, M.; Mizukami, H.; Kume, A.; et al. Comprehensive Metabolomic Analysis of IDH1 R132H Clinical Glioma Samples Reveals Suppression of β-oxidation Due to Carnitine Deficiency. *Sci. Rep.* **2019**, *9*, 1–11, doi:10.1038/s41598-019-46217-5.
18. Yaligar, J.; Teoh, W.W.; Othman, R.; Verma, S.K.; Phang, B.H.; Lee, S.S.; Wang, W.W.; Toh, H.C.; Gopalan, V.; Sabapathy, K.; et al. Longitudinal metabolic imaging of hepatocellular carcinoma in transgenic mouse models identifies acylcarnitine as a potential biomarker for early detection. *Sci. Rep.* **2016**, *6*, 1–9, doi:10.1038/srep20299.
19. Zoni, E.; Minoli, M.; Bovet, C.; Wehrhan, A.; Piscuoglio, S.; Ng, C.K.Y.; Gray, P.C.; Spahn, M.; Thalmann, G.N.; Kruithof-De Julio, M. Preoperative plasma fatty acid metabolites inform risk of prostate cancer progression and may be used for personalized patient stratification. *BMC Cancer* **2019**, *19*, 1–18, doi:10.1186/s12885-019-6418-2.
20. Li, S.; Gao, D.; Jiang, Y. Function, detection and alteration of acylcarnitine metabolism in hepatocellular carcinoma. *Metabolites* 2019, *9*, doi:10.3390/metabo9020036.
21. Reyes-Garcés, N.; Boyacı, E.; Gómez-Ríos, G.A.; Olkowicz, M.; Monnin, C.; Bojko, B.; Vuckovic, D.; Pawliszyn, J. Assessment of solid phase microextraction as a sample preparation tool for untargeted analysis of brain tissue using liquid chromatography-mass spectrometry. *J. Chromatogr. A* **2021**, *1638*, 11–13, doi:10.1016/j.chroma.2020.461862.
22. Stryjak, I.; Warmuzińska, N.; Bogusiewicz, J.; Łuczykowski, K.; Bojko, B. Monitoring of the influence of long-term oxidative stress and ischemia on the condition of kidneys using solid-phase microextraction chemical biopsy coupled with liquid chromatography–high-resolution mass spectrometry. *J. Sep. Sci.* **2020**, *43*, 1867-1878, doi:10.1002/jssc.202000032.
23. Lendor, S.; Hassani, S.A.; Boyaci, E.; Singh, V.; Womelsdorf, T.; Pawliszyn, J. Solid Phase Microextraction-Based Miniaturized Probe and Protocol for Extraction of Neurotransmitters from Brains in Vivo. *Anal. Chem.* **2019**, *91*, 4896–4905, doi:10.1021/acs.analchem.9b00995.
24. Han, S.; Liu, Y.; Cai, S.J.; Qian, M.; Ding, J.; Larion, M.; Gilbert, M.R.; Yang, C. IDH mutation in glioma: molecular mechanisms and potential therapeutic targets. *Br. J. Cancer* 2020, *122*, 1580-1589, doi: 10.1038/s41416-020-0814-x.
25. Carracedo, A.; Cantley, L.C.; Pandolfi, P.P. Cancer metabolism: Fatty acid oxidation in the limelight. *Nat. Rev. Cancer* **2013**, *13*, doi:10.1038/nrc3483.
26. Fack, F.; Tardito, S.; Hochart, G.; Oudin, A.; Zheng, L.; Fritah, S.; Golebiewska, A.; Nazarov, P. V; Bernard, A.; Hau, A.; et al. Altered metabolic landscape in IDH -mutant gliomas affects phospholipid, energy, and oxidative stress pathways . *EMBO Mol. Med.* **2017**, *9*, 1681–1695, doi:10.15252/emmm.201707729.
27. Hu, X.; Martinez-Ledesma, E.; Zheng, S.; Kim, H.; Barthel, F.; Jiang, T.; Hess, K.R.; Verhaak, R.G.W. Multigene signature for predicting prognosis of patients with 1p19q co-deletion diffuse glioma. *Neuro. Oncol.* **2017**, *19*, 786–795, doi:10.1093/neuonc/now285.
28. Wu, T.; Zheng, X.; Yang, M.; Zhao, A.; Li, M.; Chen, T.; Panee, J.; Jia, W.; Ji, G. Serum lipid alterations identified in chronic hepatitis B, hepatitis B virus-associated cirrhosis and carcinoma patients. *Sci. Rep.* **2017**, *7*, doi:10.1038/srep42710.
29. Huang, D.; Li, T.; Li, X.; Zhang, L.; Sun, L.; He, X.; Zhong, X.; Jia, D.; Song, L.; Semenza, G.L.; et al. HIF-1-mediated suppression of acyl-CoA dehydrogenases and fatty acid oxidation is critical for cancer progression. *Cell Rep.* **2014**, *8*, doi:10.1016/j.celrep.2014.08.028.